GABA METABOLISM
IN POSTHARVEST STRESS RESPONSES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Rujira Deewatthanawong

January 2011

GABA METABOLISM

IN POSTHARVEST STRESS RESPONSES

Rujira Deewatthanawong, Ph. D.

Cornell University 2011

γ-Aminobutyric acid (GABA) synthesis tends to be regulated in an organ- or tissue-specific manner, and many studies suggest that it accumulates in plants under a variety of stress conditions. However, very little is known about GABA metabolism in fruit during postharvest stresses. The objective of this dissertation study was to determine GABA metabolism in response to postharvest stresses using chilling injury inducing conditions and elevated CO_2 treatments. The dissertation reports studies on the effects of cold storage of tomato, and elevated CO_2 concentrations on strawberry and tomato on patterns of GABA accumulation. Glutamate decarboxylase (GAD) activity, GABA transaminase (GABA-T) activity, and gene expression analysis of enzymes in the GABA shunt were analyzed. The correlation between antioxidant metabolism and GABA metabolism in cold stored tomatoes was also investigated.

In tomato, GABA concentrations increased only in sensitive lines after cold storage at 3 °C for 28 d. Higher GABA concentrations were associated with lower GABA-T activity and lower expression of genes encoding succinic semialdehyde dehydrogenase (SSADH) and succinic semialdehyde reductase (SSR). Hydrogen peroxide (H_2O_2) accumulated during cold storage, but tolerant lines showed a more efficient antioxidant system as indicated by a decline in H_2O_2 during ripening at 20 ^oC, higher activity of ascorbate peroxidase (APX) during cold storage and ripening, higher peroxidase (POX) activity during ripening, and higher gene expression of superoxide dismutase (*SOD*) during cold storage.

GABA concentrations decreased in breaker fruit during storage but increased in red fruit, when treated with 10% CO_2. Greater GABA accumulation in red fruit was associated with higher CO_2 injury in fruit of that maturity stage. GABA concentrations decreased when transferred to air. CO_2 treatment was associated with higher gene expression of *GAD2* and *GAD3* in both stages, but an increase was greater in breaker fruit than red fruit. CO_2 treatment altered GABA degradation as shown by decreased GABA-T activity in both stages, but to greater extent in red fruit, as well as decreased succinic semialdehyde reductase 1 (*SSR1*) gene expression in red fruit, and decreased of *SSR2* expression in both maturity stages.

A study in strawberry cultivars with different tolerance to postharvest treatment with 20% CO_2 showed that CO_2 treatment induced GABA production, but the accumulation was not associated with sensitivity of the fruit to high CO_2 treatment as indicated by fermentation product accumulation.

The results suggest that GABA metabolism in fruit responds differently than in model systems that used intact plant organs and in which accumulation of GABA in response to stress is rapid. In postharvest systems, accumulation is delayed, if it occurs at all. The specific role of GABA in postharvest responses therefore remains uncertain.

BIOGRAPHICAL SKETCH

Rujira Deewatthanawong was a native of Chachoengsao, Thailand. After graduating from Daddarunee high school in Chachoengsao, she moved to Bangkok where she went to college. She spent 3.5 years to complete her Bachelor of Science degree in Agricultural Technology (Agronomy) from King Mongkut Institute of Technology Ladkrabang. Before going to graduate school, she worked as a temporary government employee at Department of Agricultural Extension for 5 months. She then furthered her studies at Kasetsart University, obtaining a Masters degree in Horticulture. Her Masters Thesis and Special Problem focused on senescent spotting in banana and chilling injury in mangosteen, respectively. After completion of her Masters degree in 1999, she started her academic career as a researcher at Thailand Institute of Scientific and Technological Research (TISTR). While working at TISTR, she received the Agobiotechnology training award from Japan International Cooperation Agency in 2001 to do research training at Kobe University, Japan.

Rujira was awarded the Royal Thai Government Scholarship in 2003 to pursue her Ph.D. at Cornell University. She joined the Watkins lab in summer 2005 to work on postharvest stresses with focus on γ-aminobutyric acid (GABA). She had conducted research to study GABA metabolism in response to postharvest stresses in tomato, strawberry, and apple. Additional financial support was provided by a Department of Horticulture assistantship, a Smock Scholarship, and an International Students and Scholars Office Grant.

After completion of her degree, Rujira will return to her position at Department of Agricultural Technology, TISTR.

This dissertation is dedicated to the loving memory of my dear parents,

Jaroon and Saiyud Choehom.

ACKNOWLEDGMENTS

I would like to thank my advisor, Professor Christopher B. Watkins for his patience, guidance and endless support. I would also like to extend my thanks to my committee members Dr. James J. Giovannoni and Dr. Timothy L. Setter for their generous support and suggestions. I thank Adirek Rugkong and members of the Watkins lab for their friendship and assistance. Special thanks to Jackie Nock who was always there to help. My most sincere appreciation goes to all my friends at Cornell University and in Ithaca.

Special thanks go to Prasit Deewatthanawong for his help, support and encourangement. I also wish to thank my sister, Vongduean Jai-Aoon, and her family for their love and support. My thanks also go to Dr. Anawat Suwanagul and Sodsri Neamprem at Thailand Institute of Technological Research for their kind support.

This research was supported in part by the Cornell University Agricultural Experiment Station, federal formula funds, Project NE 1036, received from the Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture. I was supported by the Royal Thai government, a Department of Horticulture assistantship, a Smock Scholarship, and an International Students and Scholars Office Grant.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACC; 1-aminocyclopropane-1-carboxylic acid

Ala; alanine

APX; ascorbate peroxidase

ATP; adenosine triphosphate

BSA; bovine serum albunin

CA; controlled atmosphere

CaM; calmodulin

CAT; catalase

cDNA; complementary deoxyribonucleic acid

CI; chilling injury

CO_2; carbon dioxide

d; day

dNTPs; deoxynucleotide triphosphates

DPA; diphenylamine

DTT; dithiothreitol

EA; ethyl acetate

EDTA; ethylenediaminetetraacetic acid

g; gram

GABA; gamma aminobutyric acid

GAD; glutamate decarboxylase

GABA-T; gamma aminobutyric acid transaminase

GABA-TK; α-kutarate-dependent gamma aminobutyric acid transaminase

GABA-TP; pyruvate-dependent gamma aminobutyric acid transaminase

GDH; glutamate dehydrogenase

GHB; gamma hydroxybutyric acid

GHBDH; gamma–hydroxybutyrate dehydrogenase

GR; glyoxylate reductase

GS/GOGAT; glutamine synthetase/glutamate synthase

h; hour

IL; introgression line

kg; kilogram

L; liter

M; molar

MAP; modified atmosphere packaging

1-MCP; 1-methylcyclopropene

min; minute

mM; millimolar

mmol; millimole

μM; micromolar

mg; milligram

mL; milliliter

μL; microliter

NAD^+; Nicotinamide adenine dinucleotide

$NADP^+$; Nicotinamide adenine dinucleotide phosphate

NBT; nitroblue tetrazolium

NMR; nuclear magnetic resonance

O_2; oxygen

PCR; polymerase chain reaction

PLP; pyridoxal phosphate

PMSF; phenylmethylsulfonyl fluoride

POX; peroxidase

ProT; proline transporter

PVP; polyvinylpyrrolidone

RNA; ribonucleic acid

ROS; reactive oxygen species

RT-PCR; reverse transcription polymerase chain reaction

SOD; superoxide dismutase

SSA; succinic semialdehyde

SSADH; succinic semialdehyde dehydrogenase

SSR; succinic semialdehyde reductase

TCA; tricarboxylic acid

v; volume

LIST OF SYMBOLS

α; alpha

γ; gamma

μ; micro

^{o}C; Celsius degree

CHAPTER 1

INTRODUCTION

γ- Aminobutyric acid or 4-aminobutyric acid (GABA), a four-carbon non-protein amino acid, is found in virtually all living organisms. The presence of this amino acid was first found in plant tissues (Hulme and Arthington, 1950), but it has been extensively studied in vertebrates as the major inhibitory neurotransmitter in the central nervous system (Robert, 1988; Kinnersley and Turano, 2000). Although the role of GABA in plant is still unclear, numerous studies have suggested its role in plant response to biotic and abiotic stresses. GABA levels in plants are normally low but increase in response to a variety of environmental stresses such as salinity, anoxia, hypoxia, drought, heat, and chilling (Streeter and Thompson, 1972a; Wallace et al., 1984; Tsushida and Murai, 1987a; Mayer et al., 1990a; Aurisano et al., 1995; Kinnersley and Lin, 2000; Zushi and Matsuzoe, 2007a). A variety of possible roles of GABA have been discussed including roles in biochemical pH-stat, the tricarboxylic acid (TCA) cycle bypass, nitrogen storage, plant development, carbon metabolism, plant defense against insects, osmoregulation, and also functions as a signaling molecule (Breitkreuza et al., 1999; Shelp et al., 1999; Kinnersley and Turano, 2000; Bouche et al., 2003c; MacGregor et al., 2003; McLean et al., 2003; Bouche and Fromm, 2004a). Recently, GABA has been suggested to have beneficial effects on human health as a natural relaxant (Abdou et al., 2006), and an antihypertensive agent (Antonaccio and Taylor, 1977; Abe et al., 1995; Yamakoshi et al., 2007; Shimada et al., 2009; Yoshimura et al., 2010), and it has been studied in tomato breeding selections (Saito et al., 2008) and in postharvest treatments (Makino et al., 2008).

1.1 The GABA shunt metabolic pathway

Glutamate, a major precursor of GABA, can be derived from the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle and the catalytic activity of glutamate dehydrogenase (GDH). GABA is metabolized via the GABA shunt which bypasses two steps of the TCA cycle affording an alternative pathway for glutamate to enter the TCA cycle. The GABA shunt is composed of three enzymes: the cytosolic glutamate decarboxylase (GAD), and the mitochondrial enzymes GABA transaminase (GABA-T) and succinate dehydrogenase (SSADH) (Bown and Shelp, 1989). GABA synthesis via the GABA shunt is thought to be the predominant pathway; however, GABA can also be derived from putrescine (Flores and Filner, 1985b; Rastogi and Davies, 1989) through the reactions of the enzymes diamine oxidase and γ-aminobutyraldehyde dehydrogenase (Trossat et al., 1997)

1.1.1 Glutamate decarboxylase

GAD catalyses the decarboxylation of glutamate, yielding CO_2 and GABA. GAD is a ubiquitous enzyme in prokaryotes and eukaryotes, but only plant GAD has been shown to have a calmodulin (CaM) binding domain (Baum et al., 1993). There are two models of GAD stimulation, one mediated by Ca^{2+}/CaM and the other by pH. Most recent experiments have focused on Ca^{2+}/CaM-induced GAD. Expression patterns of GAD mRNA and protein accumulation in different petunia organs suggested that GAD levels in plants are regulated by transcriptional and post transcriptional processes (Chen et al., 1994). Calmodulin has been shown to stimulate GAD activity by binding to a C-terminal of GAD.

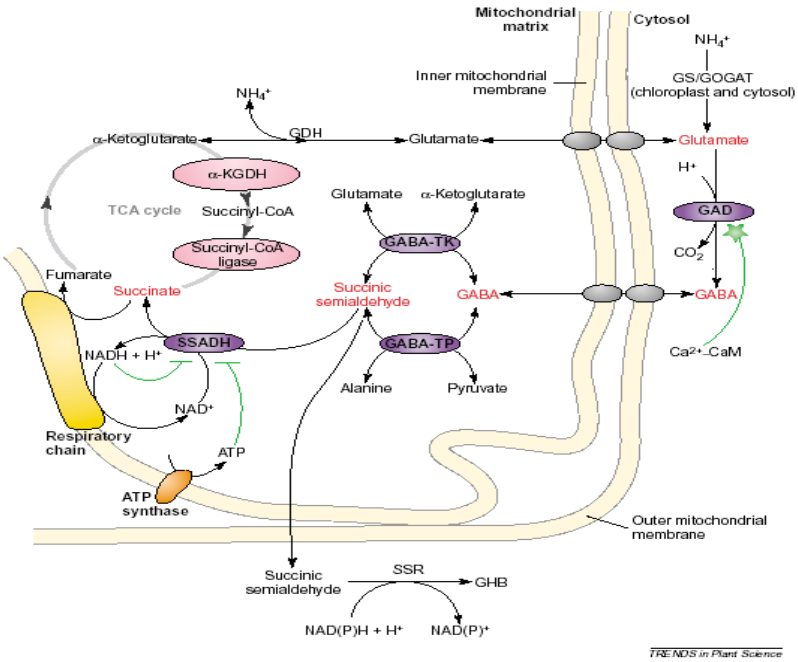


Figure 1.1 The GABA shunt pathway (Bouche and Fromm, 2004a).

Many studies have shown that CaM binding to GAD plays an important role in modulating the catalytic activity of GAD *in vitro* (Ling et al., 1994; Arazi et al., 1995; Aurisano et al., 1995; Snedden et al., 1995a; Baum et al., 1996b; Cholewa et al., 1997; Turano and Fang, 1998; Yun and Oh, 1998; Zik et al., 1998). Expression of a mutant GAD lacking the CaM-binding domain resulted in an abnormal regulation of GAD activity which was associated with extremely high GABA and low glutamate levels indicating the important of CaM binding to GAD *in vivo* (Baum et al., 1996b). The results from the immunodetection of native GAD complexes of the tobacco mutants are consistent with the 3-D structure of the CaM-binding domain of the petunia GAD, which suggested the role of the CaM-binding domain in controlling the formation or the stability of the GAD protein complex (Baum et al., 1996b; Yap et al., 2003). cDNAs encoding Ca^{2+}/CaM-dependent GADs in both dicotyledons and monocots such as petunia, tomato, tobacco, fava bean, and rice, have been isolated and

characterized (Shelp et al., 1999; Bouche et al., 2004b; Oh et al., 2005a). However, a study of rice cDNAs encoding two isoforms of GAD, *OsGAD1* and *OsGAD2*, showed 69% identity to each other and *in vitro* CaM-binding assay of the calmodulin-binding domain of plant GADs demonstrated that only *OsGAD1* can bind to bovine CaM, indicating the lack of an authentic calmodulin-binding domain at the C-terminus (Akama et al., 2001). The identification of the differential expression of the GAD isoforms indicated that the GAD isoforms are expressed in tissue-dependent manner (Turano and Fang, 1998; Zik et al., 1998; Akama et al., 2001). Antisense GAD tomatoes accumulated glutamate and had slightly lower GABA levels, but differences in GABA between transgenic and non-transgenic were not significant (Kisaka et al., 2006b). These findings reveal that proper regulation of the enzyme GAD is important for plant development and amino acid metabolism.

1.1.2 GABA transaminase

GABA is transaminated by mitochondrial GABA-T to form succinic semialdehyde. It has been shown that plant pyruvate-dependent GABA-T prefers GABA as an amino acid donor and pyruvate as an amino acid acceptor. In other organisms, GABA-T is specific for 2-oxoglutarate as the amino acid acceptor, while the amino acid donor can be either GABA or β–alanine, depending on the organism (Breitkreuz and Shelp, 1995; Van Cauwenberghe and Shelp, 1999; Van Cauwenberghe et al., 2002). Pyruvate-dependent GABA-T seems to be more abundant in plants as no study has shown the existence of 2-oxoglutarate-dependent GABA-T in *Arabidopsis*, and the instability of 2-oxoglutarate-dependent GABA-T in tobacco during purification (Van Cauwenberghe and Shelp, 1999). Previous study demonstrated that *Arabidopsis* GABA-T (*At*GABA-T) had only pyruvate-dependent activity, but not 2-oxoglutarate-dependent activity (Van Cauwenberghe et al., 2002). In *Arabidopsis* mutant, the loss of *POP2* gene encoding a GABA-T leads to a large

increase in levels of GABA in flowers but the increase is limited in other organs, suggesting different types of GABA-T which probably have different specific functions (Palanivelu et al., 2003). A recent biochemical study reported that *At*GABA-T utilized both puruvate in the reversible reaction and glyoxylate in the irreversible reaction (Clark et al., 2009a). The rice *Osl2* gene encoding pyruvate-dependent GABA-T has been reported as a novel senescence-associated gene that is upregulated during leaf senescence (Ansari et al., 2005a). A pathogen induced *OsGABA-T* was identified in rice leaves infected with blast fungus (Wu et al., 2006). Akhiro et al. (2008b) suggested a correlation between GABA catabolism and α-ketoglutarate-dependent GABA-T activity in tomato fruit, whereas the more recent study showed no α-ketoglutarate-dependent GABA-T activity in tomato cell-free extracts (Clark et al., 2009a; Clark et al., 2009b). Three tomato GABA-T genes were identified by Clark et al. (2009b). Their study indicated that all the isoforms had both pyruvate- and glyoxylate-dependent activities, and the three isoforms were localized to different subcellular compartments including the mitochondria, cytosol, and plastid.

1.1.3 Succinic semialdehyde dehydrogenase

Succinic semialdehyde is converted to succinate by the enzyme succinic semialdehyde dehydrogenase (SSADH). In this reaction, succinate and NADH are produced. Succinate then enters the the TCA cycle. *Arabidopsis* SSADH was the first cloned from plants (Busch and Fromm, 1999), and the biochemical study in developing soybean cotyledons showed that SSADH in plants is localized in mitochondria (Breitkreuz and Shelp, 1995). Plant SSADH is shown to be negatively regulated by NADH and ATP showing the feedback regulation of the rate of substrate production for the respiratory chain by the GABA shunt (Busch and Fromm, 1999; Busch et al., 2000). Environmental stresses cause a change in mitochondrial metabolism including levels of NADH and ATP, which not only affect the production

of succinate via SSADH, but also the backward reaction from succinic semialdehyde to GABA (Wigge et al., 1993; Shelp et al., 1995; Busch and Fromm, 1999). The *ssadh* knock out plants exhibited severe necrotic lesions and accumulated reactive oxygen species (ROS) when exposed to stresses (Bouche et al., 2003a).

1.1.4 Succinic semialdehyde reductase

Succinic semialdehyde reductase (SSR), which catalyzes the reduction of succinic semialdehyde (SSA) to gamma-hydroxybutyrate (GHB), has been identified as one of the NADPH-dependent aldehyde reductases (Andriamampandry et al., 1998; Maitre et al., 2000). Most of the studies have focused extensively on GHB as a neuromodulator of the mammalian brain. Studies show accumulation of GHB in plants response to O_2 deficiency and a novel γ–hydroxybutyrate dehydrogenase (GHBDH) of *Arabidopsis* was identified which provide a better understanding of its function in the plant system. It has been suggested that SSA catabolism, which involves SSADH and GHBDH activities, are regulated by redox balance (Allan et al., 2003; Breitkreuz et al., 2003a). Recently, another isoform of *Arabidopsis* GHBDH was reported and renamed as glyoxylate reductase (*AtGR1*, and *AtGR2*) (Hoover et al., 2007a; Hoover et al., 2007b; Simpson et al., 2008). Simpson et al. (2008) found that *AtGR1* was localized to the cytosol, while *AtGR2* was localized to plastids. The study also demonstrated that both isoforms catalyzed the conversion of glyoxylate to glycolate as well as SSA to GHB, but the affinity for SSA is lower than glyoxylate. SSA is known as a mitochondrial intermediate of the GABA shunt, but mitochondrial GRs have not been found. However, the possible involvement of GRs in the detoxification of SSA was suggested by Allan at al. (2008a). Their research demonstrated that GHB accumulation in *Arabidopsis* and tobacco plants is a response to abiotic stresses, and that accumulation was associated with the expression of *GR1* and *GR2* genes (Allan at al., 2008a).

1.2 Possible functions of GABA in plants

1.2.1 pH regulation

Increased Ca^{2+} and H^{+} levels under stress conditions activate GAD activity, and GABA production. Because this reaction is proton consuming, it has been hypothesized that GABA synthesis plays a role in cytosolic pH regulation. This hypothesis was supported by the results from a number of studies demonstrating that plant GADs have the maximum activity in the acidic range, and that cell acidification stimulates an increase in GABA concentrations (Satya Narayan and Nair, 1990a; Carroll et al., 1994; Crawford et al., 1994; Janzen et al., 2001). A study in asparagus mesophyll cells indicated that treatment reducing cytosolic pH using hypoxia and addition of weak acids stimulated the accumulation of both intracellular and extracellular GABA. Determination of the cytosolic location of the fluorescent pH probe confirmed that GABA synthesis is involved in cellular pH regulation (Crawford et al., 1994). However, earlier studies showed that the greatest GABA synthesis was found after large cytoplasmic acidification has ceased (Roberts et al., 1992; Shelp et al., 1999). Investigation of the CaM binding domain of plant GADs provided strong evidence for Ca^{2+}/CaM induced GAD activity. Another line of evidence supporting this hypothesis is the results from GAD purification from fava bean, soybean and petunia, which demonstrated higher GAD activity at pH 7, not pH 5.8, in the presence of Ca^{2+} or CaM (Arazi et al., 1995; Snedden et al., 1995a; Cholewa et al., 1997). Thus, plant GADs can be classified as a Ca^{2+}/CaM- and acidic pH-dependent GADs.

1.2.2 The TCA cycle bypass

The GABA shunt is a bypass of the Krebs cycle between 2-ketoglutarate and succinate, which is essentially regulated to maintain normal plant development but its role in plants is not clearly understood. It has been suggested that the GABA shunt provides carbon skeletons to replenish carboxylic acids of the TCA cycle (Bouche and

Fromm, 2004b). However, the GABA bypass is energetically less favourable (1 NADH) than the direct oxidation of 2-ketoglutarate to succinate by the TCA cycle (1 NADH and 1 ATP). Suppression of a gene encoding the β-subunit of succinyl CoA ligase in tomatoes showed only a little effect on photosynthesis, respiration and plant growth. Interestingly, the label redistribution experiment provides another line of evidence that the GABA shunt functions as the TCA cycle bypass as radioactivity from L-glutamate was recovered in GABA (Studart-Guimarães et al., 2007). Stress conditions decrease respiration rates and the NAD/NADH ratio, competitively inhibiting succinic semialdehyde dehydrogenase activity and cause accumulation of succinic semialdehyde, which in turn inhibits GABA transaminase resulting in GABA accumulation (Wigge et al., 1993; Shelp et al., 1995; Busch and Fromm, 1999). Fait et al. (2007a) suggested that an increase in carbon flux through the GABA shunt occurs during stress conditions.

1.2.3 Compatible osmolyte

The existence of a GABA transporter was confirmed by growing *Arabidopsis* on medium containing GABA as a sole nitrogen source. The proline transporter (ProT) family was known to be compatible solute transporters which also transport GABA, glycine betaine and other stress-related compounds. The transport of radiolabeled GABA by ProT2 supported that ProT2 function as a GABA transporter (Breitkreuza et al., 1999; Schwacke et al., 1999; Grallath et al., 2005). ProT2 were found ubiquitously in all plant organs but its expression was highly induced under water or salt stresses suggesting its role in compatible solute distribution (Rentsch et al., 1996; Raymond and Smirnoff, 2002). In addition, high concentration of GABA was found to stabilize isolated thylakoids under freezing and salt stresses (Heber et al., 1971). GABA with its protective properties under stress conditions has been reported but its specific role as osmoprotectant is unclear.

1.2.4 Transient nitrogen storage

Glutamate, a product of the glutamine synthetase (GS)/NADH-dependent glutamate synthase (NADH-GOGAT) pathway, is a precursor for the synthesis of many amino acids including GABA. This concept was supported by reduced levels of glutamate in transgenic plants overexpressing GAD (Baum et al., 1996b). GABA accumulation was suggested to be related to the depletion of glutamate under stresses or conditions that inhibit glutamine synthesis or increase protein degradation (Scott-Taggart et al., 1999). Sugar levels in senescing leaves decrease due to decreased photosynthesis and accumulation of GABA may play an important role in producing intermediates of the TCA cycle (Bown and Shelp, 1997). A recent study on a novel senescence-associated gene encoding GABA-pyruvate transminase showed that its expression was upregulated during rice leaf senescence, suggesting an anaplerotic role for GABA (Ansari et al., 2005a). The hypothesis that GABA also acts as a long-distance signal in the regulation of nitrate uptake was supported by an experiment in which an exogenous GABA and glutamine were supplied to roots. The study showed a correlation between GABA in phloem exudate and nitrate uptake in short and long-term experiments as well as an increased expression of BnNRT2 gene encoding nitrate transporter (Beuve et al., 2004; Le Deunff et al., 2005). Asparagine, glutamine and GABA were found to be the major amino acid content in harvested tomato fruit demonstrating that the degraded proteins and released nitrogen were transiently stored as asparagines, glutamine and GABA in tomato fruit during cell expansion stage (Baldet et al., 2002). GABA was also found one of the most abundant N compounds in phloem exudates of spruce grown on acidic soil (Schraml et al., 2002).

1.2.5 Plant development

Use of a transgenic approach has shown that the GABA shunt is required for normal plant development. Transgenic tobacco plants expressing a truncated petunia

GAD lacking the CaM-binding domain showed abnormal morphology with extremly high levels of GABA due to an abnormal regulation of GAD activity (Baum et al., 1996b). The GAD knockout mutant prevented GABA accumulation in *Arabidopsis* under normal growth conditions and in response to heat stress; however, abnormal development was not observed (Bouche et al., 2004b). The *Arabidopsis* T-DNA knockout mutants of SSADH demonstrated that the *ssadh* mutants were sensitive to UV and heat which were associated with increased levels of hydrogen peroxide and cell death. A study in double mutants of genes encoding GABA-T and SSADH (*pop2 ssadh* mutants) revealed accumulation of the GABA shunt intermediates (SSA and GHB) caused accumulation of peroxides and impaired development (Ludewig et al., 2008a). These findings suggested a role of GABA shunt in restricting the accumulation of ROS (Bouche et al., 2003b). *Arabidopsis* SSR knockout mutants showed significant accumulation of GBH in SSR-deficient mutants and VGB, a GABA-T inhibitor, reduced GHB levels and improved plant growth by preventing the accumulation of hydrogen peroxide and cell death (Fait et al., 2005b).

GABA has been identified as a key pollen-tube guidance signal (Bouche et al., 2003b; Palanivelu et al., 2003). GABA concentrations are higher at the position closer to the ovaries, thus GABA degradation is required to maintain GABA gradient in order to guide the pollen to the ovule. *Arabidopsis POP2* gene, encoding GABA-T, was shown to be involved in the formation of GABA gradient, which provided appropriate targetting for pollen tubes. The *POP2* mutant showed elevated GABA levels, which in turn eliminated the GABA gradient resulting in the fertility defect in the mutant (Ma, 2003; Palanivelu et al., 2003). Microarray analysis suggested cross-talks of the expression patterns of the pollination/fertilization and stress responses. Most pollination/fertilization repressed genes were induced by dehydration, including GABA-A receptor epsilon-like subunit gene. In addition, a putative GABA-A

transaminase is up-regulated by pollination/fertilization but down-regulated by dehydration (Lan et al., 2005).

A study in soybean hypocotyl tissue demonstrated that the rapid GABA synthesis in response to mechanical stress was associated with the rapid growth inhibition (Bown and Zhang, 2000). NMR analysis in root cultures of *Datura stramonium* suggested that de-differentiation of root tissue was accompanied by the increased levels of GABA. This change can also be induced by other factors such as phytohormone treatment and Ca^{2+} deficiency (Fliniaux et al., 2004).

GABA also stimulated ethylene biosynthesis. Exogenous GABA was found to increase the rate of ethylene production in excised sunflower tissues, which was associated with increases in ACC synthase mRNA levels, ACC levels, ACC oxidase levels, and ACC oxidase acitivity (Kathiresan et al., 1997).

Biochemical studies of tomato fruit development found that GABA accumulated in tomato fruit before the breaker stage and then decreased shortly after the breaker stage (Rolin et al., 2000; Mounet et al., 2007; Akihiro et al., 2008b; Saito et al., 2008). Akihiro et al. (2008b) also reported the positive correlation between GABA concentrations and expression of *SlGAD2* and *SlGAD3*, and the negative correlation between GABA concentrations and α-ketoglutarate-dependent GABA-T activity.

1.2.6 GABA as a signaling molecule

There is evidence supporting a proposed role of GABA as a signaling molecule in plants. Researchers have hypothesized that plants respond to wounding and insect attack by increasing GABA levels. The increased GABA levels decreased the survival of larvae and shortened the time that larvae needed to pupate (Ramputh and Brown, 1996). In *Arabidopsis*, an increase in putrescine accumulation and a decrease in spermine levels were observed after wounding. Putrescine synthesized upon wounding

was suggested to be catalized via putrescine oxidase and pyrroline dehydrogenase activities to produce GABA (Flores and Filner, 1985a). GABA synthesis was also detected several minutes after non-wounding mechanical stimulation of crawling insect larvae indicating a signaling process induced by larval footsteps (Bown et al., 2002). In addition, genetically engineered plants overexpressing GAD prevented insect feeding and infestation in tobacco suggesting a potential alternative for insect control (Shelp et al., 2003). GABA has been shown to play a role as a signal between plants and pathogenic bacteria since GABA was found to modulate quorum sensing in *A. tumefaciens*, and transgenic plants with elevated GABA concentrations were more tolerant to pathogenic bacteria infection than were wild-type plants (Chevrot et al., 2006; Mirabella et al., 2008). There is other evidence supporting the function of GABA as a signaling molecule, including a guidance of the *Arabidopsis* pollen tube by a GABA gradient (Palanivelu et al., 2003), a signal in up-regulation of nitrate uptake in *Brassica napus* (Beuve et al., 2004), the existence of GABA transporters in *Arabidopsis* (Meyer et al., 2006).

1.3 GABA receptor

GABA functions appear to be stimulated by binding to its receptors. In the mammalian central nervous system, the receptors have been classified into three classes, $GABA_A$, $GABA_B$ and $GABA_C$. $GABA_A$ and $GABA_C$ receptors belong to a superfamily of ligand-gated ionotropic receptors, while $GABA_B$ receptors are G protein-coupled receptors (Olsen et al., 1999). Comparative sequence analysis between the *Arabidopsis* genome and the animal GABA receptors did not show genes highly homologous to those found in animals. However, the evidence of high accumulation of GABA in response to stresses (Kinnersley and Turano, 2000) and the link between GABA signaling and pollen tube growth (Palanivelu et al., 2003)

indicated that GABA could interact with specific receptors. It is possible that the lack of animal homologs may due to the evolutionary mechanisms. The finding of plant glutamate receptors (GLRs) and the analysis of the N- and C-terminals of the plant GLRs and other eukaryotic receptors demonstrated that the N-terminal regions of the plant GLRs are related to animal $GABA_B$ receptors and Ca^{2+} sensors, suggesting the possibility of GABA binding to this domain (Lam et al., 1998; Turano et al., 2001). A sequence alignment of $GABA_A$-receptor-associated protein from *Arabidopsis* and other organisms showed that the core structure is highly conserved among species (Bavro et al., 2002). A study in duckweed showed that the GABA-induced growth was inhibited by modulation of $GABA_A$ receptors using the antagonists bicuculline and picrotoxin, competitive and non-competitive inhibitors. The use of Baclofen, an analog of the putative GABA, increased GABA-mediated promotion of duckweed growth (Kinnersley and Lin, 2000). These findings suggest that the putative GLRs may function as GABA-like receptors in plants.

1.4 GABA and sensitivity of plants to stresses

GABA accumulates in response to many stresses including salinity, anoxia, hypoxia, drought, heat, and chilling (Streeter and Thompson, 1972a; Wallace et al., 1984; Tsushida and Murai, 1987a; Mayer et al., 1990a; Aurisano et al., 1995; Kinnersley and Turano, 2000; Zushi and Matsuzoe, 2007a). The possible correlation between GABA metabolism and tolerance of plants to stresses is still unclear. However, studies on GABA in frost-resistant and frost-sensitive cultivars in barley and wheat reveal differences between the two groups (Mazzucotelli et al., 2006). GABA-related amino acids were higher in frost-resistant cultivars. An increase in GAD activity caused glutamate depletion in frost-sensitive cultivars, while glutamate levels were retained in frost-resistant cultivars. In addition, GABA degradation via the

GABA shunt only occurred in a frost-resistant genotype. A recent study on salinity stress in tomatoes demonstrates that the effect on GABA promoting was greatest in the sensitive cultivar, and the effect of the stress on its accumulation did not occur during the early development stages (Saito et al., 2008).

Little is known about GABA in response to postharvest stresses. However, an increase in GABA was observed in of cold stored cherimoya fruits during the last stage of storage (Escribano and Merodio, 2001), in 20% CO_2 treated cherimoya fruit (Merodio et al., 1998a), in tomatoes stored in modified atmospheres (Makino *et al.*, 2008), and in controlled atmosphere stored pears (Franck et al., 2007a; Pedreschi et al., 2009).

1.5 Postharvest stress treatments

Many steps in the postharvest handling process of fresh produce can cause a variety of abiotic stresses to commodities. Controlled atmosphere (CA), cold storage and a combination of both are well-established technologies widely used to prolong and maintain quality of many kinds of horticultural produce. Under these environmental conditions, stored produce may experience abiotic stresses such as hypoxia or chilling stresses, which in turn cause metabolic changes. Because GABA is produced and accumulated in response to biotic or abiotic stresses, its accumulation could be a metabolic marker for stressed plant cells. However, only limited information is available relating to GABA metabolism and defense mechanisms in stored produce after harvest.

1.5.1 Postharvest oxidative stress

Postharvest storage protocols that cause stresses such as chilling and hypoxia that disrupt the cellular homeostasis of cells result in an increase of the production of ROS and oxidative stress occurs when the production of ROS exceeds the scavenging

capacity of the plant cell. During stresses, ROS serve as second messengers that mediate responses to stresses by activating or suppressing a number of transcription factors via ROS signal transduction pathway (Apel and Hirt, 2004). Studies have shown the roles of the GABA shunt in antioxidant defense systems. Analysis of *ssadh Arabidopsis* mutants have been shown to accumulate high levels of hydrogen peroxide (Bouche et al., 2003a), while double *pop2 ssadh* mutants did not accumulate peroxides (Ludewig et al., 2008a). SSADH knockout mutants also showed higher GHB concentrations, and treatment with the GABA-T inhibitor could prevent the accumulation of ROS and GHB, providing another line of evidence that the GABA shunt plays a role in controlling levels of ROS in plants under stresses (Fait et al., 2005b). However, the opposite effect of GHB has been suggested to function naturally as an endogenous protective agent (Mamelak, 1989).

1.5.2 Cold storage

Cold storage of some commodities is restricted by chilling injury, which is a disorder of crops of tropical and subtropical origin when stored at low temperature (Wang, 1990). It was thought that the main target of chilling injury is cell membranes, including alteration in membrane structure and lipid composition. The ratio of saturated to unsaturated fatty acids is considered to be a major factor suggesting the sensitivity of plants to the chilling stress (Quinn, 1988). Chilling-sensitive plants have a higher proportion of saturated fatty acids and plants can become acclimated by slow exposure to colder temperatures, which increases the ratio of unsaturated fatty acids to saturated fatty acids in the membranes (Quinn, 1988). However, low temperature is also known to induce or alter the expression of many genes, indicating that there are other necessary mechanisms involving chilling stress responses (Anderson et al., 1994; Seki et al., 2001; Kreps et al., 2002; Provart et al., 2003). GABA accumulation in stressed plants may be another mechanism that plays a crucial role as a defense

mechanism against the damage caused by chilling stress. The concentration of calcium of cold stored produce, which has a role in intracellular signalling, has been associated with the incidence of chilling injury symptoms. Chilling injury symptoms were minimal in fruit parts, where higher concentrations of calcium were found (Chaplin and Scott, 1980; Chang et al., 2001; Hewajulige et al., 2003). The calcium signalling system plays an important role in pollen tube growth, cell development and differentiation, hormonal signal transduction, programmed cell death, and adaptation to various stress factors (Knight, 2000; Medvedev, 2005). Increased cytosolic calcium stimulates GAD activity and GABA accumulation (Kinnersley and Turano, 2000). These lines of evidence suggest a possible connection of GABA metabolism and chilling injury.

1.5.3 Controlled atmosphere storage

Low O_2 or elevated CO_2 atmospheres can reduce respiration and ethylene production rates, delay ripening and senescence processes of the stored produce (Kader, 2003). However, if the conditions result in O_2 deficient conditions, plants respond to the hypoxia by altering cellular metabolism. Studies have shown that the formation of ROS and anaerobic fermentation are associated with exposure to hypoxia, although there is no clear conclusion whether sensitivity or tolerance to the stress are due to energy depletion or increased oxidative damage (Fukao and Bailey-Serres, 2004; Beiley-Serres and Chang, 2005). The genes that are induced in response to O_2 deprivation in *Arabidopsis* involve various metabolisms, including carbohydrate catabolism, glycolysis, anaerobic fermentation, lipid metabolism, ethylene synthesis, auxin-mediated processes, and calcium and ROS-mediated signal transduction. A number of genes encoding transcription factors and signal transduction components have been found to increase in response to hypoxia, indicating complexity of cellular defence mechanisms against hypoxia (Paul et al., 2003; Branco-Price et al., 2005; Liu

et al., 2005). A Rho of plant (Rop) G-protein was reported to be activated during hypoxia resulting in an increase in hydrogen peroxide, which was considered a second messenger in signal transduction pathways. The formation of ROS leads to an increase in transcription and activation of genes involved in anaerobic metabolism (Fukao and Bailey-Serres, 2004; Beiley-Serres and Chang, 2005). The composition of the amino acid pool is changed during hypoxia, specifically the accumulation of alanine, GABA and putrescine, indicating that amino acid metabolism is involved in maintaining cellular homeostasis (Reggiani and Bertani, 2003).

A postharvest study in cherimoya fruit showed that GABA content in fruit stored at 20% CO_2 was higher compared with untreated fruit, but the accumulation decreased when transferred to air (Merodio et al., 1998b). High CO_2 concentrations was shown to induce genes encoding a ripening-regulated heat shock protein and GAD (Rothan et al., 1997). In addition, GABA accumulation was found in brown tissue in pears under elevated CO_2 and low O_2 condition (Franck et al., 2007a; Pedreschi et al., 2009). GABA concentrations increased when tomatoes were stored under modified atmospheres (11% O_2 and 9% CO_2) at 30 ^{o}C (Makino et al., 2008).

REFERENCES

Abdou, A.M., Higashiguchi, S., Horrie, K., Kim, M., Hatta, H., Yokogoshi, H., 2006. Relaxation and immunity enhancement effects of γ-aminobutyric acid (GABA) administration in humans. Biofactors 26, 201-208.

Akama, K., Akihiro, T., Kitagawa, M., Takaiwa, F., 2001. Rice (*Oryza sativa*) contains a novel isoform of glutamate decarboxylase that lacks an authentic calmodulin-binding domain at the C-terminus. Biochim Biophys Acta. 1522, 143-150.

Akihiro, T., Koike, S., Tani, R., Tominaga, T., Watanabe, S., Iijima, Y., Aoki, K., Shibata, D., Ashihara, H., Matsukura, C., Akama, K., Fujimura, T., Ezura, H., 2008. Biochemical mechanism on GABA accumulation during fruit development in tomato. Plant Cell Physiol. 49, 1378-1389.

Allan, W.L., Peiris, C., Bown, A.W., Shelp, B.J., 2003. Gamma-hydroxybutyrate accumulates in green tea and soybean sprouts in response to oxygen deficiency. Can. J. Plant Sci. 83, 951-953.

Allan, W.L., Simpson, J.P., Clark, S.M., Shelp, B.J., 2008. γ-Hydroxybutyrate accumulation in Arabidopsis and tobacco plants is a general response to abiotic stress: putative regulation by redox balance and glyoxylate reductase isoforms. J. Exp. Bot. 59, 2555-2564.

Anderson, M.D., Prasad, T.K., Martin, B.A., Stewart, C.R., 1994. Differential gene expression in chilling-acclimated maize seedlings and evidence for the involvement of abscisic acid in chilling tolerance. Plant Physiol. 105, 331-339.

Andriamampandry, C., Siffert, J.-C., Schmitt, M., Garnier, J.-M., Staub, A., Muller, C., Gobaille, S., Mark, J., Maitre, M., 1998. Cloning of a rat brain succinic

semialdehyde reductase involved in the synthesis of the neuromodulator gamma-hydroxybutyrate. Biochem. J. 334, 43-50.

Ansari, M.I., Lee, R.H., Chen, S.C.G., 2005. A novel senescence-associated gene encoding γ-aminobutyric acid (GABA):pyruvate transaminase is upregulated during rice leaf senescence. Physiol. Plantarum 123, 1-8.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Ann. Rev. Plant Biol. 55, 373-399.

Arazi, T., Baum, G., Snedden, W.A., Shelp, B.J., Fromm, H., 1995. Molecular and biochemical analysis of calmodulin interactions with the calmodulin-binding domain of plant glutamate decarboxylase. Plant Physiol. 108, 551-561.

Aurisano, N., Bertani, A., Reggiani, R., 1995. Involvement of calcium and calmodulin in protein and amino acid metabolism in rice roots under anoxia. Plant Cell Physiol. 36, 1525-1529.

Baldet, P., Devaux, C., Chevalier, C., Brouquisse, R., Just, D., Raymond, P., 2002. Contrasted responses to carbohydrate limitation in tomato fruit at two stages of development. Plant Cell Environ. 25, 1639–1649.

Baum, G., Chen, Y., Arazi, T., Takatsuji, H., Fromm, H., 1993. A plant glutamate decarboxylase containing a calmodulin binding domain. Cloning, sequence, and functional analysis. J. Biol. Chem. 268, 19610-19617.

Baum, G., Lev-Yadun, S., Fridmann, Y., Arazi, T., Katsnelson, H., Zik, M., Fromm, H., 1996. Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. EMBO J. 15, 2988-2996.

Bavro, V.N., Sola, M., Bracher, A., Kneussel, M., Betz, H., Weissenhorn, W., 2002. Crystal structure of the GABAA-receptor-associated protein, GABARAP. EMBO Rep. 3, 183-189.

Beiley-Serres, J., Chang, R., 2005. Sensing and signalling in response to oxygen deprivation in plants and other organisms. Ann. Bot. 96, 507-518.

Beuve, N., Rispail, N., Laine, P., Cliquet, J.B., Ourry, A., Le Deunff, E., 2004. Putative role of gamma-aminobutyric acid (GABA) as a long distance signal in up-regulation of nitrate uptake in *Brassica napus* L. Plant Cell Environ. 27, 1035-1046.

Bouche, N., Fait, A., Bouchez, D., Moller, S.G., Fromm, H., 2003a. Mitochondrial succinic-semialdehyde dehydrogenase of the γ-aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. PNAS 100, 6843-6848.

Bouche, N., Lacombe, B., Fromm, H., 2003b. GABA signaling: a conserved and ubiquitous mechanism. Trends Cell Biol. 13, 607-610.

Bouche, N., Fait, A., Zik, M., Fromm, H., 2004. The root-specific glutamate decarboxylase (GAD1) is essential for sustaining GABA levels in *Arabidopsis*. Plant Mol. Biol. 55, 315-325.

Bouche, N., Fromm, H., 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9, 110-115.

Bown, A.W., Shelp, B.J., 1989. The metabolism and physiological roles of 4-aminobutyric acid. Biochem. J. 8, 21-25.

Bown, A.W., Shelp, B.J., 1997. The metabolism and functions of γ-aminobutyric acid. Plant Physiol. 115, 1-5.

Bown, A.W., Zhang, G., 2000. Mechanical stimulation, 4-aminobutyric acid (GABA) synthesis, and growth inhibition in soybean hypocotyl tissue. Can. J. Bot. 78, 119-123.

Bown, A.W., Hall, D.E., MacGregor, K.B., 2002. Insect footsteps on leaves stimulate the accumulation of 4-aminobutyrate and can be visualized through increased

chlorophyll fluorescence and superoxide production. Plant Physiol. 129, 1430-1434.

Branco-Price, C., Kawaguchi, R., Ferriera, R., Bailey-Serres, J. 2005. Genome-wide analysis of transcript abundance and translation in *Arabidopsis* seedlings subjected to oxygen deprivation. Ann. Bot. 96, 647-660.

Breitkreuz, K.E., Shelp, B.J., 1995. Subcellular compartmentation of the 4-aminobutyrate shunt in protoplasts from developing soybean cotyledons. Plant Physiol. 108, 99-103.

Breitkreuz, K.E., Allan, W.L., Cauwenberghe, O.R.V., Jakobs, C., Talibi, D., Andre, B., Shelp, B.J., 2003. A novel γ-hydroxybutyrate dehydrogenase. Identification and expression of an *Arabidopsis* cDNA and potential role under oxygen deficiency. J. Biol. Chem. 278, 41552-41556.

Breitkreuza, K.E., Shelp, B.J., Fischer, W.N., Schwacke, R., Rentsch, D., 1999. Identification and characterization of GABA, proline and quaternary ammonium compound transporters from *Arabidopsis thaliana*. FEBS Lett. 450, 280-284.

Busch, K., Piehler, J., Fromm, H., 2000. Plant succinic semialdehyde dehydrogenase: dissection of nucleotide binding by surface plasmon resonance and fluorescence spectroscopy. Biochem. 39, 10110–10117.

Busch, K.B., Fromm, H., 1999. Plant succinic semialdehyde dehydrogenase. Cloning, purification, localization in mitochondria, and regulation by adenine nucleotides. Plant Physiol. 121, 589-597.

Carroll, A.D., Fox, G.G., Laurie, S., Phillips, R., Ratcliffe, R.G., Stewart, G.R., 1994. Ammonium assimilation and the role of γ-aminobutyric acid in pH homeostasis in carrot cell suspensions. Plant Physiol. 106, 513-520.

Chang, M.-Y., Chen, S.-L., Lee, C.-F., Chen, Y.-M., 2001. Cold-acclimation and root temperature protection from chilling injury in chilling-sensitive mungbean (*Vigna radiata* L.) seedlings. Bot. Bull. Acad. Sin. 42, 53-60.

Chaplin, G.R., Scott, K.J., 1980. Association of calcium in chilling injury susceptibility of stored avocados. HortSci. 15, 514-515.

Chen, Y., Baum, G., Fromm, H., 1994. The 58-kilodalton calmodulin-binding glutamate decarboxylase is a ubiquitous protein in petunia organs and its expression is developmentally regulated. Plant Physiol. 106, 1381-1387.

Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B.J., 2006. GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. PNAS 103, 7460-7464.

Cholewa, E., Cholewinski, A.J., Sheen, J., Snedden, W.A., Bown, A.W., 1997. Cold shock-stimulated γ-aminobutyrate synthesis is mediated by an increase in cytosolic Ca^{2+} not by an increase cytosolic H^{+}. Can. J. Bot. 75, 375-382.

Clark, S.M., Di Leo, R., Dhanoa, P.K., Van Cauwenberghe, O.R., Mullen, R.T., Shelp, B.J., 2009a. Biochemical characterization, mitochondrial localization, expression, and potential functions for an *Arabidopsis* γ-aminobutyrate transaminase that utilizes both pyruvate and glyoxylate. J. Exp. Bot. 60, 1743-1757.

Clark, S.M., Di Leo, R., Van Cauwenberghe, O.R., Mullen, R.T., Shelp, B.J., 2009b. Subcellular localization and expression of multiple tomato γ-aminobutyrate transaminases that utilize both pyruvate and glyoxylate. J. Exp. Bot. 60, 2701-2712.

Crawford, L.A., Bown, A.W., Breitkreuz, K.E., Guinel, F.C., 1994. The synthesis of γ-aminobutyric acid in response to treatments reducing cytosolic pH. Plant Physiol. 104, 865-871.

Escribano, M.I., Merodio, C., 2001. Limited ammonium assimilation in cherimoya fruit stored at chilling temperature. Acta Hort. 533, 311-312.

Fait, A., Yellin, A., Fromm, H., 2005. GABA shunt deficiencies and accumulation of reactive oxygen intermediates: insight from *Arabidopsis* mutants. FEBS Lett. 579, 415-420.

Fait, A., Fromm, H., Walter, D., Galili, G., Fernie, A.R., 2007. Highway or byway: the metabolic role of the GABA shunt in plants. Trends Plant Sci. 13, 14-19.

Fliniaux, O., Mesnard, F., Grandic, S.R., Baltora-Rosset, S.R., Bienaimé, S., Robins, R.J., Fliniaux, M.-A., 2004. Altered nitrogen metabolism associated with de-differentiated suspension cultures derived from root cultures of *Datura stramonium* studied by heteronuclear multiple bond coherence (HMBC) NMR spectroscopy. J. Exp. Bot. 55, 1053-1060.

Flores, H.E., Filner, P., 1985. Polyamine catabolism in higher plants: characterization of pyrroline dehydrogenase. Plant Growth Regul. 3, 277-291.

Franck, C., Lammertyn, J., Tri Ho, Q., Verboven, P., Verlinden, B., Nicolaï, B.M., 2007. Browning disorders in pear fruit. Postharvest Biol. Technol. 43, 1-13.

Fukao, T., Bailey-Serres, J., 2004. Plant responses to hypoxia is survival a balancing act? Trends Plant Sci. 9, 449-456.

Grallath, S., Weimar, T., Meyer, A., Gumy, C., Suter-Grotemeyer, M., Neuhaus, J.M., Rentsch, D., 2005. The AtProT family: compatible solute transporters with similar substrate specificity but differential expression patterns. Plant Physiol. 137, 117-126.

Heber, U., Tyankova, L., Santarius, K.A., 1971. Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. Biochim Biophys Acta 241, 578-592.

Hewajulige, I., Wilson Wijeratnam, R., Wijesundera, R., Abeysekere, M., 2003. Fruit calcium concentration and chilling injury during low temperature storage of pineapple. J. Sci. Food Agric. 83, 1451-1454.

Hoover, G.J., Prentice, G.A., Merrill, A.R., Shelp, B.J., 2007a. Kinetic mechanism of *Arabidopsis* glyoxylate reductase: studies of initial velocity, dead-end inhibition and product inhibition. Can. J. Bot. 95, 896-902.

Hoover, G.J., Van Cauwenberghe, O.R., Breitkreuz, K.E., Clark, S.M., Merrill, A.R., Shelp, B.J., 2007b. Characteristics of an *Arabidopsis* glyoxylate reductase: general biochemical properties and substrate specificity for the recombinant protein, and developmental expression and implications for glyoxylate and succinic semialdehyde metabolism *in planta*. Can. J. Bot. 85, 883-895.

Hulme, A.C., Arthington, W., 1950. γ–Aminobutyric acid and β–alanine in plant tissue. Nature 165, 716-717.

Janzen, D.J., Allen, L.J., MacGregor, K.B., Bown, A.W., 2001. Cytosolic acidification and γ-aminobutyric acid synthesis during the oxidative burst in isolated *Asparagus sprengeri* mesophyll cells. Can. J. Bot. 79, 438-443.

Kader, A.A., 2003. Physiology of CA treated produce. Acta Hort. 600, 349-354.

Kathiresan, A., Tung, P., Chinnappa, C.C., Reid, D.M., 1997. γ-Aminobutyric acid stimulates ethylene biosynthesis in sunflower. Plant Physiol. 115, 129-135.

Kinnersley, A.M., Lin, F., 2000. Receptor modifiers indicate that 4-aminobutyric acid (GABA) is a potential modulator of ion transport in plants. Plant Growth Regul. 32, 65-76.

Kinnersley, A.M., Turano, F.J., 2000. Gamma aminobutyric acid (GABA) and plant responses to stress. Crit. Rev. Plant Sci. 19, 479-509.

Kisaka, H., Kida, T., Miwa, T., 2006. Antisense suppression of glutamate decarboxylase in tomato (*Lycopersicon esculentum* L.) results in accumulation of glutamate in transgenic tomato fruits. Plant Biotech. 23, 267-274.

Knight, H., 2000. Calcium signalling during abiotic stress in plants. Int.Rev.Cytol. 195, 1011-1020.

Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., Harper, J.F., 2002. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol. 130, 2129-2141.

Lam, H.-M., Chiu, J., Hsieh, M.-H., Meisel, L., Oliveira, I.C., Shin, M., Coruzzi, G., 1998. Glutamate-receptor genes in plants. Nature 396, 125-126.

Lan, L., Li, M., Lai, Y., Xu, W., Kong, Z., Ying, K., Han, B., Xue, Y., 2005. Microarray analysis reveals similarities and variations in genetic programs controlling pollination/fertilization and stress responses in rice (*Oryza sativa* L.). Plant Mol. Biol. 59, 151-164.

Ling, V., Snedden, W.A., Shelp, B.J., Assmann, S.M., 1994. Analysis of a soluble calmodulin binding protein from fava bean roots: identification of glutamate decarboxylase as a calmodulin-activated enzyme. Plant Cell 6, 1135-1143.

Liu, F., Vantoai, T., Moy, L., Bock, G., Linford, L.D., Quackenbush, J., 2005. Global transcription profiling reveals novel insights into hypoxic response in *Arabidopsis*. Plant Physiol. 137, 1115-1129.

Ludewig, F., Huser, A., Fromm, H., Beauclair, L., Bouche, N., 2008. Mutants of GABA transaminase (*POP2*) suppress the severe phenotype of succinic semialdehyde dehydrogenase (ssadh) mutants in *Arabidopsis*. PLoS One 3, e3383.

Ma, H., 2003. Plant reproduction: GABA gradient, guidance and growth. Curr. Biol. 13, 834-836.

MacGregor, K.B., Shelp, B.J., Peiris, S., Bown, A.W., 2003. Overexpression of glutamic acid decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. J. Chem. Ecol. 29, 2177-2182.

Maitre, M., Andriamampandry, C., Kemmel, V., Schmidt, C., Hode, Y., Hechler, V., Gobaille, S., 2000. Gamma-hydroxybutyric acid as a signaling molecule in brain. Alcohol 20, 277-283.

Makino, Y., Soga, N., Oshita, S., Kawagoe, Y., Tanaka, A., 2008. Stimulation of γ-aminobutyric acid production in vine-ripe tomato (*Lycopersicon esculentum* Mill.) fruits under modified atmospheres. J. Agri. Food Chem. 56, 7189-7193.

Mamelak, M., 1989. Gamma-hydroxybutyrate: an endogenous regulator of energy metabolism. Neurosci. Biobehav. Rev. 13, 187-198.

Mayer, R.R., Cherry, J.L., Rhodes, D., 1990. Effects of heat shock on amino acid metabolism of cowpea cells. Phytochemistry 94, 796-810.

Mazzucotelli, E., Tartari, A., Cattivelli, L., Forlani, G., 2006. Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. J. Exp. Bot. 57, 3755-3766.

McLean, M.D., Yevtushenko, D.P., Deschene, A., Van Cauwenberghe, O.R., Makhmoudova, A., Potter, J.W., Bown, A.W., Shelp, B.J., 2003. Overexpression of glutamic acid decarboxylase in transgenic tobacco plants confers resistance to the northern root-knot nematode. Mol. Breed. 11, 277-285.

Medvedev, S.S., 2005. Calcium signalling system in plants. Russ. J. Plant Physiol. 52, 249-270.

Merodio, C., Munoz, M.T., Cura, B.D., Buitrago, D., Escribano, M.I., 1998. Effect of high CO_2 level on the titres of γ-aminobutyric acid, total polyamines and some

pathogenesis-related proteins in cherimoya fruit stored at low temperature. J. Exp. Bot. 49, 1339-1347.

Meyer, A., Eskandari, S., Grallath, S., Rentsch, D., 2006. AtGAT1, a high affinity transporter for γ-aminobutyric acid in *Arabidopsis thaliana*. J. Biol. Chem. 281, 7197-7204.

Mirabella, R., Rauwerda, H., Struys, E.A., Jakobs, C., Triantaphylides, C., Haring, M.A., Schuurink, R.C., 2008. The *Arabidopsis her1* mutant implicates GABA in E-2-hexenal responsiveness. Plant J. 53, 197-213.

Oh, S.-H., Choi, W.-G., Lee, I.-T., Yun, S.J., 2005. Cloning and characterization of a rice cDNA encoding glutamate decarboxylase. J. Biochem. Mol. Biol. 38, 595-601.

Olsen, R.W., DeLorey, T.M., Gordey, M., Kang, M.H., 1999. GABA receptor function and epilepsy. Adv. Neurol. 79, 499-510.

Palanivelu, R., Brass, L., Edlund, A.F., Preuss, D., 2003. Pollen tube growth and guidance is regulated by *POP2*, an *Arabidopsis* gene that controls GABA levels. Cell 114, 47-59.

Paul, A.L., Schuerger, A.C., Popp, M.P., Richards, J.T., Manak, M.S., Ferl, R.J., 2003. Hypobaric biology: *Arabidopsis* gene expression at low atmospheric pressure. Plant Physiol. 134, 215-223.

Pedreschi, R., Franck, C., Lammerteyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123-130.

Provart, N.J., Pedro Gil, P., Chen, W., Han, B., Chang, H.-S., Wang, X., Zhu, T., 2003. Gene expression phenotypes of *Arabidopsis* associated with sensitivity to low temperatures. Plant Physiol. 132, 893-906.

Quinn, P.J., 1988. Effects of temperature on cell membranes. Symp. Soc. Exp. Biol. 42, 237-258.

Ramputh, A.-I., Brown, A.W., 1996. Rapid γ-aminobutyric acid synthesis and the inhibition of the growth and development of oblique-banded leaf-roller larvae. Plant Physiol. 111, 1349-1352.

Rastogi, R., Davies, P.J., 1989. Polyamine metabolism in ripening tomato fruit I. Identification of metabolites of putrescine and spermidine. Plant Physiol. 94, 1449-1455.

Raymond, M.J., Smirnoff, N., 2002. Proline Metabolism and transport in maize seedlings at low water potential. Ann. Bot. 89, 813-823.

Reggiani, R., Bertani, A., 2003. Anaerobic amino acid metabolism. Russ. J. Plant Physiol. 50, 733-736.

Rentsch, D., Hirner, B., Schmelzer, E., Frommer, W.B., 1996. Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. Plant Cell 8, 1437-1446.

Robert, E., 1988. The establishment of GABA as a neurotransmitter. In: Squiers, R.F. (Ed.), GABA and Benzodiazepine Receptors. CRC Press, Boca Raton. pp. 1-21.

Roberts, J.K.M., Hooks, M.A., Miaullis, A.P., Edwards, S., Webster, C., 1992. Contribution of malate and amino acid metabolism to cytoplasmic pH regulation in hypoxic maize root tips studied using nuclear magnetic resonance spectroscopy. Plant Physiol. 98, 480-487.

Rothan, C., Duret, S., Chevalier, C., Raymond, P., 1997. Suppression of ripening-associated gene expression in tomato fruits subjected to a high CO_2 concentration. Plant Physiol. 114, 255.263.

Saito, T., Matsukura, C., Sugiyama, M., Watahiki, A., Ohshima, I., Iijima, Y., Konishi, C., Fujii, T., Inai, S., Fukuda, N., Nishimura, S., Ezura, H., 2008. Screening for γ-aminobutyric acid (GABA)-rich tomato varieties. J. Japan. Soc. Hort. Sci. 77, 242-250.

Satya Narayan, V., Nair, P.M., 1990. Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plant. Phytochemistry 29, 365-375.

Schraml, C., Herschbach, C., Eiblmeier, M., Rennenberg, H., 2002. Consequences of elevated CO_2, augmented nitrogen-deposition and soil type on the soluble nitrogen and sulphur in the phloem of beech (*Fagus sylvatica*) and spruce (*Picea abies*) in a competitive situation. Physiol. Plantarum 115, 258-266.

Schwacke, R., Grallath, S., Breitkreuz, K.E., Stransky, E., Stransky, H., Frommer, W.B., Rentsch, D., 1999. LeProT1, a transporter for proline, glycine betaine, and γ-amino butyric acid in tomato pollen. Plant Cell 11, 377-392.

Scott-Taggart, C.P., Van Cauwenberghe, O.R., McLean, M.D., Shelp, B.J., 1999. Regulation of gamma-aminobutyric acid synthesis in situ by glutamate availability. Physiol. Plantarum 106, 363-369.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., Shinozaki, K., 2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell 13, 61-72.

Shelp, B.J., Walton, C.S., Snedden, W.A., Tuin, L.G., Oresnik, I.J., Layzell, D.B., 1995. Gaba shunt in developing soybean seeds is associated with hypoxia. Physiol. Plantarum 94, 219-228.

Shelp, B.J., Bown, A.W., McLean, M.D., 1999. Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446-452.

Shelp, B.J., Van Cauwenberghe, O.R., Bown, A.W., 2003. Gamma aminobutyrate: from intellectual curiosity to practical pest control. Can. J. Bot. 81, 1045-1048.

Simpson, J.P., Di Leo, R., Dhanoa, P.K., Allan, W.L., Makhmoudova, A., Clark, S.M., Hoover, G.J., Mullen, R.T., Shelp, B.J., 2008. Identification and characterization of a plastid-localized *Arabidopsis* glyoxylate reductase isoform: comparison with a cytosolic isoform and implications for cellular redox homeostasis and aldehyde detoxification. J. Exp. Bot. 59, 2545-2554.

Snedden, W.A., Arazi, T., Fromm, H., Shelp, B.J., 1995. Calcium/calmodulin activation of soybean glutamate decarboxylase. Plant Physiol. 108, 543-549.

Streeter, J., Thompson, J.F., 1972. Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). Plant Physiol. 49, 572-578.

Studart-Guimarães, C., Fait, A., Nunes-Nesi, A., Carrari, F., Usadel, B., Fernie, A.R., 2007. Reduced expression of succinyl CoA ligase can be compensated for by an upregulation of the γ-amino-butyrate (GABA) shunt in Illuminated tomato leaves Plant Physiol. 145, 626-639.

Subbaiah, C.C., Bush, D.S., Sacks, M.M., 1998. Mitochondrial contribution to the amoxic Ca^{2+} signal in maize suspension-cultured cells. Plant Physiol. 118, 759-771.

Trossat, C., Rathinasabapathi, B., Hanson, D., 1997. Transgenically expressed betaine aldehyde dehydrogenase effieciently catalyzes oxidation of dimethylsulfoniopropionaldehyde and ω-aminoaldehydes. Plant Physiol. 113, 1457-1461.

Tsushida, T., Murai, T., 1987. Conservation of glutamic acid to γ-aminobutyric acid in tea leaves under anaerobic conditions Agric. Biol. Chem. 51, 2865-2871.

Turano, F.J., Fang, T.K., 1998. Characterization of two glutamate decarboxylase cDNA clones from *Arabidopsis*. Plant Physiol. 117, 1411-1421.

Turano, F.J., Panta, G.R., Allard, M.W., Berkum, P.V., 2001. The putative glutamate receptors from plants are related to two superfamilies of animal neurotransmitter receptors via distinct evolutionary mechanisms. Mol. Biol. Evol. 18, 1417-1420.

Van Cauwenberghe, O.R., Shelp, B.J., 1999. Biochemical characterization of partially purified GABA:pyruvate transaminase from *Nicotiana tabacum*. Phytochemistry 52, 575-581.

Van Cauwenberghe, O.R., Makhmoudova, A., McLean, M.D., Clark, S.M., Shelp, B.J., 2002. Plant pyruvate-dependent gamma-aminobutyrate transaminase: identification of an *Arabidopsis* cDNA and its expression in *Escherichia coli*. Can. J. Bot. 80, 933-941.

Wallace, W., Secor, J., Schrader, L.E., 1984. Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol. 75, 170-175.

Wang, C.Y., 1990. Chilling Injury of Horticultural Crops. CRC Press, Boca Raton, FL. pp. 313.

Wigge, B., Kroemer, S., Gardestroem, P., 1993. The redox levels and subcellular distribution of pyridine nucleotides in illuminated barley leaf protoplasts studied by rapid fractionation. Physiol. Plantarum 88, 10-18.

Wu, C., Zhou, S., Zhang, Q., Zhao, W., Peng, Y., 2006. Molecular cloning and differential expression of an gamma-aminobutyrate transaminase gene, *OsGABA-T*, in rice (*Oryza sativa*) leaves infected with blast fungus. J. Plant Res. 119, 663-669.

Yamakoshi, J., Fukuda, S., Satoh, T., Tsuji, R., Saito, M., Obata, A., Matsuyama, A., Kikuchi, M., Kawasaki, T., 2007. Antihypertensive and natriuretic effects of

less-sodium soy sauce containing γ-aminobutyric acid in spontaneously hypertensive rats. Biosci. Biotechnol. Biochem. 71, 165-173.

Yap, K.L., Yuan, T., Mal, T.K., Vogel, H.J., Ikura, M., 2003. Structural basis for simultaneous binding of two carboxy-terminal peptides of plant glutamate decarboxylase to calmodulin. J. Mol. Biol. 328, 193-204.

Yun, S.J., Oh, S.H., 1998. Cloning and characterization of a tobacco cDNA encoding calcium/calmodulin-dependent glutamate decarboxylase. Mol. Cells 8, 125-129.

Zik, M., Arazi, T., Snedden, W.A., Fromm, H., 1998. Two isoforms of glutamate decarboxylase in *Arabidopsis* are regulated by calcium/calmodulin and differ in organ distribution. Plant Mol. Biol. 37, 967-975.

Zushi, K., Matsuzoe, N., 2007. Salt stress-enhanced γ-aminobutyric acid (GABA) in tomato fruit. Acta Hort. 761, 431-436.

CHAPTER 2

ACCUMULATION OF γ-AMINOBUTYRIC ACID IN APPLE, STRAWBERRY AND TOMATO FRUIT IN RESPONSE TO POSTHARVEST TREATMENTS

ABSTRACT

The aim of the present preliminary study was to determine whether GABA accumulation occurs in apple, strawberry and tomato in response to postharvest treatments. γ-Aminobutyric acid (GABA) concentrations have been investigated in apple and strawberry fruit exposed to elevated CO_2 levels, and in tomato fruit of *L. pennellii* introgression lines in cultivated tomato (*S. lycopersicum*) stored at 3°C. In apple fruit, external CO_2 injury was much greater in fruit exposed to 2.5% and 5% CO_2 than at 1% CO_2, and injury was enhanced by an inhibitor of ethylene perception, 1-methylcyclopropene (1-MCP), and inhibited by antioxidant, diphenylamine (DPA), treatment before exposure to 5% CO_2. GABA accumulated in fruit skin to a greater extent when exposed to 2.5% and 5% CO_2 than to 1% CO_2, while 1-MCP treated fruit had enhanced GABA accumulation and DPA treated fruit less GABA accumulation compared with 5% CO_2 alone. Strawberries contained undetectable amounts of GABA at harvest, but its concentration increased markedly during storage in 20% CO_2. Tomato fruits of *L. pennellii* introgression lines stored at 3°C for 21 d showed elevated GABA concentrations, and the percentage increase over GABA concentrations at harvest was greater in fruit of introgression lines with higher susceptibility to chilling injury. Overall, the results suggest that GABA accumulations may be related to tolerance of fruit to postharvest stresses.

2.1 INTRODUCTION

γ-Aminobutyric acid or 4-aminobutyric acid (GABA) is a four-carbon non-protein amino acid that has long been of interest to plant physiologists because it accumulates in response to stress conditions such as salinity, anoxia, hypoxia, drought, heat, and chilling in a number of plant systems (Aurisano et al., 1995; Mayer et al., 1990; Wallace et al., 1984; Zushi and Matsuzoe, 2007). GABA has also been increasingly recognized as an important contributor to the C:N balance, and may be an integral part of the TCA cycle under both stress and non-stress conditions (Bouche and Fromm, 2004; Fait et al., 2007). In addition it may have roles as a regulator of cytosolic pH, protection against oxidative stress, defense against insects, as an osmoregulator and as a signaling molecule (Bouche and Fromm, 2004).

Compared with other plant systems, less is known about GABA metabolism in fruit and vegetables in response to postharvest stresses during storage. GABA concentrations decreased during early storage of cold stored cherimoya fruits, but then increased (Escribano and Merodio, 2001). GABA accumulated in cherimoya fruits stored at 20% CO_2, but then decreased after fruits were transferred to air (Merodio et al., 1998b). Makino et al. (2008) found that GABA concentrations increased in tomato fruits stored in modified atmospheres, while a metabolic profiling study showed increasing GABA concentrations in controlled atmosphere-stored pear fruit that developed flesh browning (2007b; Pedreschi et al., 2009).

The objective of the current study was to determine a number of fruit types under controlled atmosphere and cold conditions as part of an initial study on GABA metabolism in harvested products. We tested apple and strawberry fruit under elevated CO_2 concentrations, and tomato fruit under chilling injury-inducing conditions. The 'Empire' apple in elevated CO_2 was chosen because of the sensitivity of the fruit to external CO_2 injury (Fawbush et al., 2008), the strawberry because of its

tolerance to elevated CO_2 atmospheres (Watkins et al., 1999; Fernández-Trujillo et al., 1999), and the tomato because of its sensitivity to chilling injury (King and Ludford, 1983; Malacrida et al., 2006).

2.2 MATERIALS AND METHODS

2.2.1 Apple fruit

'Empire' fruit were harvested from a commercial orchard in western New York with a history of fruit susceptibility to CO_2 injury. Fruit of uniform size and free from blemishes were picked during the commercial harvest period (Blanpied and Silsby, 1992) and transported to the laboratory on the day of harvest. For each experiment, fruit were randomized to provide 18 lots of 100 fruit per replicate. Three lots were treated with 1 μL L^{-1} 1-MCP as described by Watkins et al. (2000), and another three lots were treated with 1 g L^{-1} DPA (Shield DPA 15%, Pace International, Wenatchee, WA) by submerging fruit for 1 min.

Fruit were cooled to 0.5°C for 6 h and then each fruit lot was then transferred to 19 L glass jars (40 fruit per jar) and connected to a flow-through system that provided humidified premixed gas mixtures of 1, 2.5 or 5% CO_2 (in 2% O_2). The 1-MCP and DPA treated fruit were stored in 5% CO_2 (in 2% O_2). Flow rates of the gases were maintained at 200 mL min^{-1}, and atmospheres were monitored at least once daily by gas chromatography (Fisher Gas Partitioner, model 1200, Fisher Scientific, Springfield, NJ). There were three replicates for each CO_2 atmosphere and treatment. Ten fruit per replicate were sampled at 0, 1, 2, 4 and 8 weeks of exposure to CO_2, quickly peeled and the peel tissue frozen in liquid nitrogen and stored at -80°C until used for GABA analyses. The remaining fruit were used for assessment of external CO_2 injury at day 56 of treatment plus 7 d at 20°C.

2.2.2 Strawberry fruit

'Northeaster' strawberries were harvested at the red ripe stage at a local commercial farm and transported to the Cornell University Postharvest Laboratory where they were sorted to eliminate damaged fruit, and selected for uniform size and color. About 45 fruit were placed into 1.9 L Mason glass jars. After allowing fruit temperatures to equilibrate to the appropriate storage temperature for 2 h, the jars were connected to flow boards and exposed to either air or 20% CO_2 in air at 3°C (Shin et al., 2008). Samples were collected on day 0, 5, 10, 15 and 20. On each time point, jars were removed in triplicate, and fruit were cut quickly into liquid nitrogen. The tissues were stored at -80°C until used for GABA analyses.

2.2.3 Tomato fruit

A population of *Solanum pennellii* introgression lines in the background of *S.lycopersicum* (M 82) (Eshed and Zamir, 1994) were grown under field conditions in Freeville, NY. On the basis of a preliminary screening of 76 introgression lines, three lines (2-4, 2-6 and 8-1-2) that were more tolerant to chilling stress, and three lines (3-2, 7-4 and 7-5-5) that were chilling sensitive were harvested and stored at 3°C for 21 d. Six fruit of each line were sampled while still cold. Pericarp tissue was frozen in liquid nitrogen and stored at -80°C until used for GABA analyses. A further six fruit were transferred to 20°C for assessment of CI after 1 d at 20°C. Pitting and decay incidence were visually evaluated using a four-grade scoring system where 0 = no pitting or decay; 1 = a few scattered pits or slightly decay; 2 = more than 1% but less than 5% coverage; 3 = more than 5% but less than 25% coverage; and 4 = more than 25% coverage.

2.2.4 GABA determination

Frozen pericarp tissue was ground in liquid nitrogen to a fine powder and around 0.4 g of ground sample was added into a microtube containing 0.4 mL

methanol and vortexed. The tube was left at room temperature for 10 min and then vacuum dried using a Vacufuge Concentrator 5301 (Eppendorf, Hamburg, Germany). Then, 1 mL of 70 mM lanthanum chloride was added, the sample shaken for 15 min, and centrifuged at 13,000 x *g* for 5 min. 800 μL of supernatant was transferred to a new tube and 160 μL of 1 M potassium hydroxide was added, and the tube was then shaken for 5 min followed by centrifugation at 13,000 x *g* for 5 min. The supernatant was collected for analysis.

The GABA concentration was measured by using the enzymatic reaction of GABase, a mix of GABA-T and SSADH. The 1 mL reaction consisted of 0.6 mM $NADP^+$, 0.1 unit of GABase, 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α-ketoglutarate and 550 μL of diluted extract. The absorbance at 340 nm was read before and 10 min after adding α-ketoglutarate using a Spectronic Genesys 5 spectrophotometer (Spectronic Instruments, Waltham, MA).

2.3 RESULTS

2.3.1 Apple fruit

External CO_2 injury in 'Empire' apples occurs within the first 28-42 d of storage, injury was higher at 2.5% and 5% CO_2 than at 1% CO_2 (Table 2.1). Injury was absent when fruit were treated with DPA, while 1-MCP further enhanced CO_2 injury.

GABA accumulated in fruit stored in 1% CO_2 only slowly over the 56 d storage period, while GABA accumulated to reach maximum concentrations by day 28 in fruit stored in 2.5 and 5% CO_2 (Fig 2.1A). GABA concentrations in the elevated CO_2 treated fruit then declined to levels similar to those in fruit stored at 1% CO_2. For ease of comparison, GABA accumulations in fruit stored in 5% CO_2, either alone or after DPA and 1-MCP treatment are shown separately (Fig 2.1B). DPA treatment

markedly decreased the accumulation of GABA that was found in fruit stored in 5% CO_2. In 1-MCP treated fruit, GABA accumulation was similar to that of the untreated fruit at day 28, but continued to increase rather than decline by day 56.

Table 2.1 External CO_2 injury (%) in 'Empire' apples exposed to 1, 2.5 or 5% CO_2, or to 5% CO_2 after treatment of fruit with 1 μL L^{-1} 1-MCP or 1 g L^{-1} DPA, for 56 d at 0.5°C. Injury was evaluated after fruit were kept at 20°C for a further 7 d.

Averages of all data (n=3) were analyzed ANOVA.

Treatment	External CO_2 injury (%)
1% CO_2	7c
2.5% CO_2	56b
5% CO_2	70b
5% CO_2 + DPA	0d
5% CO_2 + 1-MCP	97a

Means followed by the same letters are not significantly different at $P \leq 0.05$.

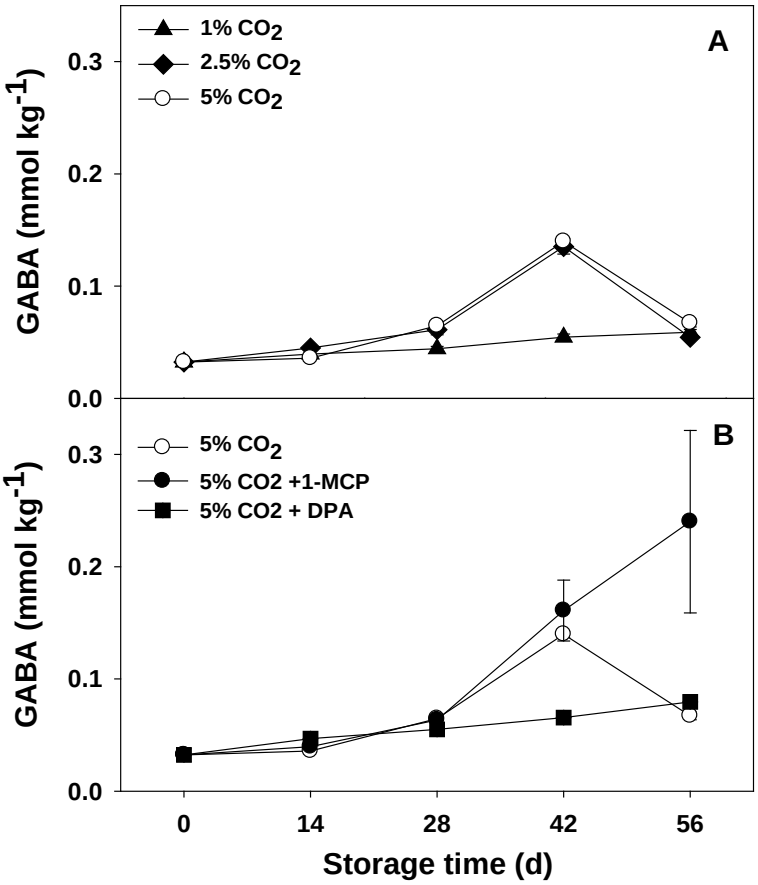


Figure 2.1 GABA concentrations in the peel tissues of 'Empire' apples (A) stored in 1%, 2.5% or 5% CO_2 for 56 d, or (B) stored in 5% CO_2 alone, or after treatment with 1 µL L^{-1} 1-MCP or 1 g L^{-1} DPA. Means are shown with standard error bars except when smaller than the symbol.

2.3.2 Strawberry fruit

'Northeaster' strawberry fruit contained very low or undetectable amounts of GABA at harvest and no GABA accumulation occurred in fruit stored in air (Fig 2.2). In contrast, GABA accumulated in fruit that were stored in 20% CO_2, although increases were relatively small by day 5 of treatment. By day 20, however, GABA concentrations were over 300 times higher than those of air-stored fruit.

2.3.3 Tomato fruit

Pitting, a symptom of CI, was not detected in introgression lines 2-4, 2-6 and 8-1-2 while all fruit of lines 3-2, 7-4 and 7-5-5 developed pitting symptoms after 21 d at $3^{o}C$ (Fig 2.3A). Decay, sometimes a secondary symptom of CI, also differed in the tomato lines, but less consistently. GABA concentrations were variable across lines at harvest, and were not related to subsequent CI sensitivity (Fig 2.3B). GABA accumulated in all fruit lines during cold storage; a relationship was observed between chilling sensitivity and percent increase in GABA. The increase in GABA levels (% of control) of lines 2-4, 2-6, 8-1-2, 3-2, 7-4 and 7-5-5 was 193%, 125%, 147%, 535%, 235% and 257%, respectively.

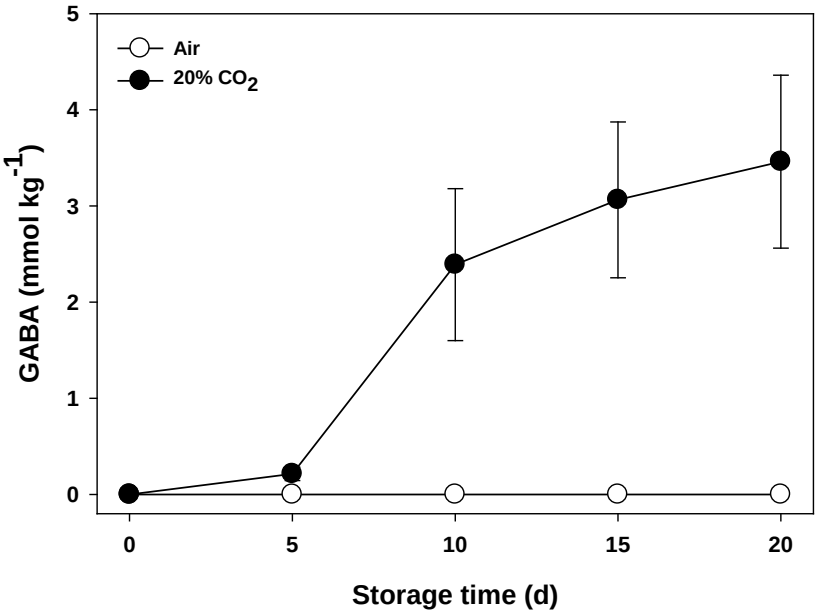


Figure 2.2 GABA concentrations in 'Northeaster' strawberry fruit stored in air or CO_2 for 20 d. Means are shown with standard error bars except when smaller than the symbol.

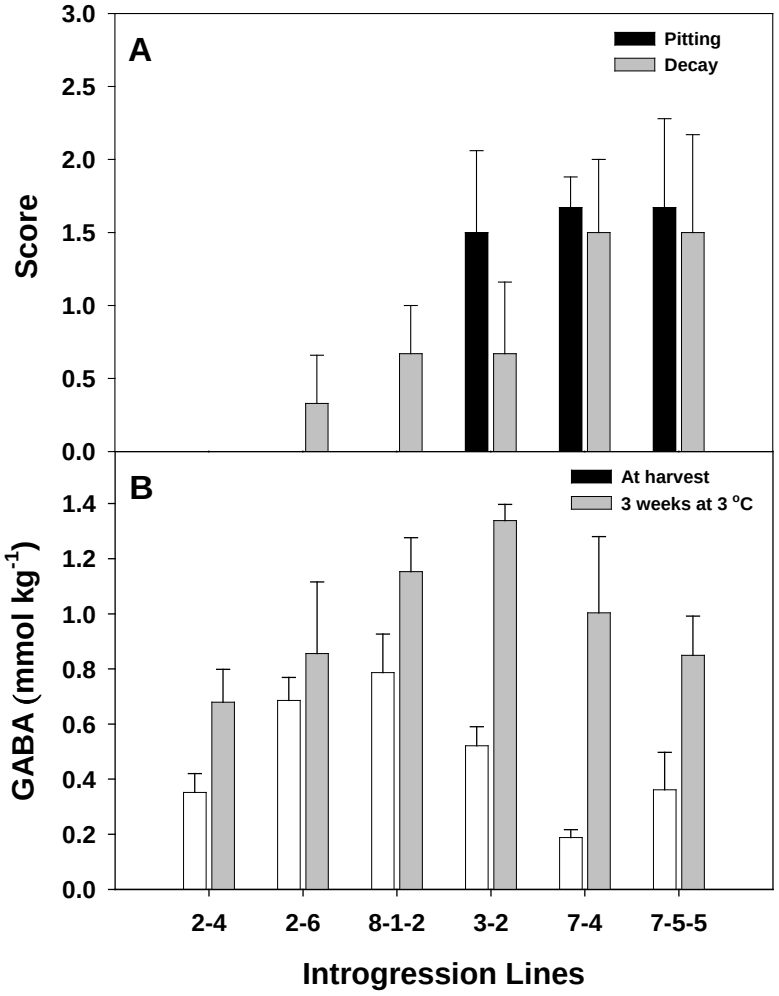


Figure 2.3 (A) Severity of surface pitting and decay of tomato introgression lines at 3°C for 21 d, and (B) GABA concentrations in fruit at harvest and after storage. Means are shown with standard error bars except when smaller than the symbol.

2.4 DISCUSSION

In 'Empire' apples, external CO_2 injury is greatest when fruit are exposed to higher CO_2 concentrations, treatment of fruit with 1-MCP can enhance susceptibility of fruit injury, and DPA can prevent injury (Burmeister and Dilley, 1995; DeEll et al., 2003; Fawbush et al., 2008). The association of GABA accumulation with external CO_2 injury suggests a possible stress response in these fruit when exposed to elevated concentrations. DPA is an antioxidant used commercially to prevent development of the physiological disorder superficial scald, but it has broad spectrum effects on a number of stress-related disorders including CO_2 injury. The GABA shunt may be associated with antioxidant defense systems in controlling levels of reactive oxygen species (ROS) in plants under stresses (Bouche et al., 2003; Fait et al., 2005). No studies on GABA metabolism in apple fruit appear to be available, but Frank et al. (2007) suggested that accumulation of GABA in brown tissues of controlled atmosphere stored pear fruit was a response to anaerobic conditions in the fruit.

Strawberry fruit are tolerant of high concentrations of CO_2, and commercially are sometimes treated with elevated (15-20%) CO_2 to maintain firmness and reduce decay (Mitcham, 2004). However, cultivars vary in their tolerance to these conditions, less resistant ones accumulating fermentation products with increasing periods of exposure to high CO_2 (Watkins et al., 1999; Fernández-Trujillo et al., 1999). CO_2 treatments clearly result in stress conditions in these fruit, and therefore accumulation of GABA would be expected. While 'Northeaster' strawberries have been used in our laboratory to evaluate the effects of CO_2 on antioxidant activities of the fruit (Shin et al., 2008), no data on the accumulation of fermentation products is available. It is not known therefore, if any association between GABA and fermentation accumulation exist.

GABA accumulation in plant exposed to chilling temperatures have been reported in barley, wheat, and cherimoya (Escribano and Merodio, 2001; Mazzucotelli et al., 2006). Our study showed that tomato introgression lines with higher sensitivity to chilling stress had greater increases in GABA concentrations compared with the less susceptible lines.

In this preliminary study, I have examined three systems to investigate postharvest stress effects on GABA accumulation. The results indicate that GABA accumulates under postharvest stress conditions and the accumulation may be related to sensitivity of fruit to stresses.

REFERENCES

Aurisano, N., Bertani, A. and Reggiani, R., 1995. Involvement of calcium and calmodulin in protein and amino-acid metabolism in rice roots under anoxia. Plant Cell Physiol. 36, 1525-1529.

Blanpied, G.D., Silsby, K.J., 1992. Predicting harvest date windows of apples. Info. Bul. 221, Cornell Coop. Ext.

Bouche, N., Fait, A., Bouchez, D., Moller, S. G., Fromm, H., 2003. Mitochondrial succinic-semialdehyde dehydrogenase of the γ-aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. PNAS 100, 6843-6848.

Burmeister, D.M., Dilley, D.R., 1995. A scald-like controlled atmosphere storage disorder of Empire apples-a chilling injury induced by CO_2. Postharvest Biol. Technol. 6, 1–7.

DeEll, J.R., Murr, D.P., Wiley, L., Porteous, M.D., 2003. 1-Methylcyclopropene (1-MCP) increases CO_2 injury in apples. Acta Hortic. 600, 277–280.

Eshed, Y., Zamir, D., 1994. A genomic library of *Lycopersicon pennellii* in *L. esculentum*: A tool for fine mapping of genes. Euphytica 79, 175-179.

Fait, A., Yellin, A., Fromm, H., 2005. GABA shunt deficiencies and accumulation of reactive oxygen intermediates: insight from *Arabidopsis* mutants. *FEBS Lett.* 579, 415-420.

Fawbush, F., Nock, J.F., Watkins, C.B., 2008. External carbon dioxide injury and 1-methylcyclopropene (1-MCP) in the 'Empire' apple. Postharvest Biol. Technol. 48, 92-98.

Fernández-Trujillo, J.P., Nock, J.F., Watkins, C.B., 1999. Fermentative metabolism and organic acid concentrations in fruit of selected strawberry cultivars with different tolerances to carbon dioxide, J. Am. Soc. Hortic. Sci. 124, 696–701.

Franck, C., Lammertyna, J., Tri Ho, Q., Verboven, P., Verlinden, B., Nicolai, B. M., 2007. Browning disorders in pear fruit. Postharvest Biol. Technol. 43, 1-13.

King, M.M., Ludford, P.M., 1983. Chilling injury and electrolyte leakage in fruit of different tomato cultivars. J. Am. Soc. Hort. Sci. 108, 74-77.

Malacrida, C., Valle, E. M., Boggio, S. B., 2006. Postharvest chilling induces oxidative stress response in the dwarf tomato cultivar Micro-Tom. Physiol. Plant. 127, 10-18.

Mayer, R. R., Cherry, J. L., Rhodes, D., 1990. Effects of heat shock on amino acid metabolism of cowpea cells. Phytochemistry 94, 796-810.

Shin, Y., Ryu, J.A., Liu, R.H., Nock, J.F., Polar-Cabrera, K., Watkins, C.B., 2008. Fruit quality, antioxidant contents and activity, and antiproliferative activity of strawberry fruit stored in elevated CO_2 atmospheres. J. Food Sci. 73, S339-344.

Wallace, W., Secor, J. ,Schrader, L. E., 1984. Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol. 75, 170-175.

Watkins, C.B., Manzano-Mendez, J.E., Nock, J.F., Zhang, J.J., Maloney, K.E., 1999. Cultivar variation in response of strawberry fruit to high carbondioxide treatments. J.Sci. Food Agric. 79, 886-890.

Watkins, C.B., Nock, J.F. and Whitaker, B.D., 2000. Responses of early, mid and late season apple cultivars to postharvest application of 1-methylcyclopropene (1-

MCP) under air and controlled atmosphere storage conditions. Postharvest Biol. Technol. 19, 17-32.

Zhang, G., Bown, A.W., 1997. The rapid determination of γ-aminobutyric acid. Phytochemistry 44, 1007-1009.

Zushi, K., Matsuzoe, N., 2007. Salt stress-enhanced γ-aminobutyric acid (GABA) in tomato fruit. Acta Hort. 761, 431-435.

CHAPTER 3

γ- AMINOBUTYRIC ACID AND ANTIOXIDANT METABOLISM IN TOMATOES IN RESPONSE TO CHILLING TEMPERATURES

ABSTRACT

γ-Aminobutyric acid (GABA) and antioxidant metabolism has been studied in breeding lines that were selected from an introgression population of *Solanum pennellii* in cultivated tomato (*S. lycopersicum*) based on sensitivity to chilling injury. The objective of the present study was to compare GABA concentrations and antioxidant enzyme activities in lines with different tolerances to chilling temperature. Fruits were stored for 28 d at 3 ^{o}C, plus a shelf life of 14 d at 20 ^{o}C. After cold storage, GABA concentrations were lower in tolerant lines, but higher in sensitive lines, compared with those at harvest. Higher GABA concentrations in sensitive lines were associated with lower activity of GABA transaminase (GABA-T) and lower gene expression of succinic semialdehyde dehydrogenase (*SSADH*) and succinic semialdehyde reductase (*SSR*). Hydrogen peroxide accumulated during cold storage of all lines, but decreased during ripening at 20 ^{o}C only in tolerant lines. Fruits of the tolerant lines had higher ascorbate peroxidase (APX) activity during storage and ripening, higher peroxidase (POX) activity during ripening after cold storage, and greater transcript levels of superoxide dismutase (*SOD*) during cold storage. Our results suggest that the chilling tolerance is associated with a functional GABA shunt and a more efficient scavenging of reactive oxygen species.

3.1 INTRODUCTION

γ- Aminobutyric acid or 4-aminobutyric acid (GABA) is a four-carbon non-protein amino acid that is found in virtually all living organisms. It was first found in plant tissues (Hulme and Arthington, 1950), but it has been most studied in vertebrates as the major inhibitory neurotransmitter in the central nervous system which helps to balance the brain activity (Robert, 1988; Kinnersley and Turano, 2000). In plant systems, GABA levels often increase in response to stresses such as salinity, anoxia, hypoxia, drought, heat, and chilling (Streeter and Thompson, 1972b; Wallace et al., 1984; Tsushida and Murai, 1987b; Mayer et al., 1990b; Aurisano et al., 1995; Kinnersley and Turano, 2000; Zushi and Matsuzoe, 2007b), but little is known about the roles of GABA in response to postharvest stresses during storage of fruits and vegetables. GABA concentrations decreased during early storage of cold stored cherimoya fruits, but then increased (Escribano and Merodio, 2001). GABA accumulated in cherimoya fruits stored at 20% CO_2, but then decreased after fruits were transferred to air (Merodio et al., 1998b), and Makino *et al.* (2008) found that GABA concentrations increased in tomato fruits stored in modified atmospheres at 30 ^{o}C. In pears, GABA concentration increased in fruits that developed flesh browning when stored in controlled atmosphere storage (Franck et al., 2007b; Pedreschi et al., 2009).

Cold storage of many horticultural commodities, especially those of tropical and subtropical origin, is restricted by chilling injury (CI). For tomato, the optimum storage temperature is 13-18 °C, and CI can occur if they are stored at temperatures below 10 °C (Thompson, 2002). Antioxidative systems are involved in resistance of plants to CI; chilling stress is thought to disrupt the cellular homeostasis of cells, resulting in an increase of the production of reactive oxygen species (ROS) that cause oxidative stress when the production of ROS exceeds the scavenging capacity of the

plant cell (Hodges et al., 1997; Mittler, 2002; Malacrida et al., 2006). ROS also serve as second messengers that mediate responses to stresses by activating or suppressing a number of transcription factors via the reactive oxygen intermediates signal transduction pathway (Apel and Hirt, 2004). ROS detoxification mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities. Superoxide is converted to H_2O_2 by SOD. H_2O_2 is then detoxified by APX, and/or CAT. Thus, the balance between the ROS scavenging system and the production system is very important to limit ROS accumulation (Foyer et al., 1994; Mittler, 2002).

The GABA shunt may be associated with antioxidant defense systems in controlling levels of ROS in plants under stresses; an *Arabidopsis* mutant deficient in a gene encoding succinic semialdehyde dehydrogenase (SSADH) accumulated high levels of H_2O_2 (Bouche et al., 2003a). The *SSADH* knockout mutants also showed an increase in the level of γ-hydroxybutyrate (GHB), and treatment with the GABA-transaminase (GABA-T) inhibitor prevented accumulation of ROS and GHB (Fait et al., 2005b).

The objective of the current study was to assess the possible role of GABA and antioxidant metabolism in chilling sensitive and chilling tolerant tomatoes. A cross between *S. pennellii* and *S. lycopersicum* was first screened for responses of fruit to CI-inducing conditions, and four lines chosen for further study based on their sensitivity to injury. GABA and activities of the GABA shunt enzymes, GAD and GABA-T, were carried out during cold storage at 3 ^{o}C and during ripening of fruit at 20 ^{o}C before storage and after 28 d of storage. Analyses of gene expression of GABA shunt and antioxidant enzymes, H_2O_2, and activities of antioxidant enzymes are restricted to ripening with and without cold storage for 28 d.

3.2 MATERIALS AND METHODS

3.2.1 Fruit source and treatment

A population of *S. pennellii* introgression lines in the background of *S. lycopersicum* (Eshed and Zamir, 1994) were grown under field conditions in Freeville, NY. To screen for chilling sensitivity of the introgression lines, six fruit at the breaker stage of maturity were harvested from each of 68 lines and then transported to the Cornell postharvest laboratory in Ithaca. Fruit were sorted and stored at 3 ^{o}C for 21 d and then transferred to 20 ^{o}C for assessment of chilling symptoms. On the basis of the results of this experiment, IL 2-6 and IL11-2 were selected as tolerant lines while IL 3-4 and IL 7-4-1 as sensitive lines for further study. Tomato seeds of these introgression lines were obtained from the C. M. Rick Tomato Genetics Resource Center at UC Davis. Plants were grown in a greenhouse at Cornell University, Ithaca, NY. Fruit were harvested at the breaker stage and either kept at 20 ^{o}C for 14 d, or stored at 3 ^{o}C for up to 28 d. Fruit were sampled at 7 d intervals. At 28 d, all remaining fruit were transferred to 20 ^{o}C for 14 d. Six replicate fruit per line were used at each sampling time point. On each sampling day, fruit quality was evaluated, pericarp tissues of each fruit was sampled for glutamate decarboxylase (GAD) and GABA-T enzyme analyses, and the remaining pericarp tissue was immediately frozen in liquid nitrogen and stored at -80 ^{o}C until used.

3.2.2 Fruit quality evaluations

Fruit color was measured at 3 points on the equator of each fruit using a Minolta Chromameter CR-300 (Osaka, Japan) which was calibrated with a white calibration plate prior to use. The readings were recorded as Chroma and Hue angles.

Pitting and decay incidence was visually evaluated at each sampling time. The severity of surface pitting and decay was determined using a four-grade scoring system where 0 = no pitting or decay; 1 = a few scattered pits or slightly decay; 2 =

less than 5% coverage; 3 = more than 5% but less than 25% coverage; and 4 = more than 25% coverage.

3.2.3 GABA determination

GABA was extracted and assayed as described by Zhang and Bown (Zhang and Bown, 1997). Frozen pericarp tissue was ground in liquid nitrogen to a fine powder and around 0.4 g of ground sample was added into a microtube containing 0.4 mL methanol and vortexed. The tube was left at room temperature for 10 min and then vacuum dried using a Vacufuge Concentrator 5301 (Eppendorf, Hamburg, Germany). Then 1 mL of 70 mM lanthanum chloride was added, the sample shaken for 15 min, and centrifuged at 13,000 x *g* for 5 min. 800 μL of supernatant was transferred to a new tube and 160 μL of 1 M potassium hydroxide was added, and the tube was then shaken for 5 min followed by centrifugation at 13,000 x *g* for 5 min. The supernatant was used for analysis of the GABA concentration by the enzymatic reaction of GABase, a mix of GABA-T and SSADH. The 1 mL reaction consisted of 0.6 mM $NADP^+$, 0.1 unit of GABase, 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α-ketoglutarate and 550 μL of diluted extract. The absorbance at 340 nm was read before and 10 min after adding α-ketoglutarate using a Genesys 5 spectrophotometer (Spectronic Instruments, Waltham, MA).

3.2.4 GAD and GABAT activity assays

Five grams of fresh tomato pericarp were homogenized in 15 mL extraction buffer containing 0.1 M Tris-Cl (pH 9.1), 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 5mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM pyridoxal phosphate (PLP) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was then filtered through Miracloth and centrifuged at 24,500 x *g* at 4 ^oC for 30 min. The supernatant was used for enzyme assays.

GAD activity was assayed according to the method of Bartyzel et al. (2003a) with some modification. GAD activity was determined by incubating 50 μL of crude extract in 0.1 M potassium phosphate buffer (pH 5.8), 40 μM PLP and 3 mM L-glutamate (total volume of 400 μL). The blank contained everything except L-glutamate. The reaction was incubated for 60 min at 30 ^{o}C and 0.1 mL of 0.5 M hydrochloric acid was added to stop the reaction. The amount of GABA produced was determined as described above.

GABA-T was assayed using pyruvate as an amino group acceptor, according to the protocol of Ansari et al. (2005a). The 500 μL reaction mixture contained 50 mM Tris-Cl (pH 8.2), 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM PLP , 10% (v/v) glycerol, 16 mM GABA, 4 mM pyruvate and 200 μL crude extract. The blank contained everything except the substrates, GABA and pyruvate. The reaction was terminated after 1 h incubation at 30 ^{o}C by adding 50 μL of 40 mM sulphosalicylic acid. The amount of alanine produced was measured using alanine dehydrogenase (EC 1.4.1.1). The 1 mL reaction contained a terminated enzyme reaction, 50 mM sodium carbonate buffer (pH 10), 1.5 mM NAD^{+} and 0.02 unit of L-alanine dehydrogenase. Absorbance at 340 nm was read after 10 min incubation at 25 ^{o}C using a Spectronic Genesys 5 spectrophotometer.

3.2.5 Antioxidant enzyme activities

Five grams of frozen tomato pericarp were ground in liquid nitrogen and added to precooled 15 mL extraction buffer containing 200 mM sodium phosphate buffer (pH 7.8), 2 mM EDTA, 5% (w/v) polyvinylpyrrolidone (PVP) and 1 mM PMSF. The mixture was homogenized, filtered through Miracloth, and then centrifuged at 24,000 x *g* at 4 ^{o}C for 30 min.

Superoxide dismutase (SOD) activity was measured according to Giannopolitis and Ries (1977) with some modifications. The ability of the enzyme to inhibit light

induced conversion of nitroblue tetrazolium (NBT) to formazan was determined by illuminating a 2 mL reaction mixture containing 100 mM sodium phosphate buffer (pH 7.8), 57 μM NBT, 0.025% (v/v) Triton X-100, 0.11 mM EDTA, 0.01 M methionine, 1.13 μM riboflavin and the enzyme extract under a set of 2 fluorescent lamps (15-20W each) for 15 min. Absorbance was read at 560 nm using a DU 7400 spectrophotometer (Beckman Instrument Co., Fullerton, CA). One unit of SOD activity was defined as a point where a sample gives 50% inhibition of the NBT reaction.

Peroxidase (POX) activity was measured according to Rao *et al.* (1996). The assay mixture contained 100 mM sodium phosphate buffer (pH 6.8), 2.7 mM guaiacol, 4 mM H_2O_2 and the enzyme extract. Decreases in absorbance at 470 nm were recorded for 3 min using a DU 7400 spectrophotometer.

Ascorbate peroxidase (APX) activity was assayed using the method described by Nakano and Asada (1981) with modifications. The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 1.3 mM ascorbate, 1 mM H_2O_2 and the enzyme extract. A decrease in absorbance at 290 nm was recorded for 1 min using a DU 7400 spectrophotometer.

Catalase (CAT) activity was determined according to the method described by Boonsiri *et al.* (2007). The reaction mixture contained 12 mM H_2O_2 in 0.1 M potassium phosphate buffer (pH 7.0) and the crude extract. Enzyme activity was evaluated by monitoring the decrease of absorbance at 240 nm for 5 min using a DU 7400 spectrophotometer.

3.2.6 Protein

Protein concentrations were measured using the Bradford assay (Sigma, St Louis, MO) according to the manufacturer's protocol. The net absorbance at 595 nm of each sample was compared with the bovine serum albumin (BSA) standard curve.

3.2.7 Hydrogen peroxide

H_2O_2 was measured as described by Mondal *et al.* (2004). Five grams of frozen pericarp tissue were homogenized in 15 mL of ice cold 0.01 M potassium phosphate buffer, pH 7.0 and centrifuged at 24,000 x *g* at 4 ^{o}C for 15 min. The reaction consisted of 2 mL diluted sample in 0.01 M phosphate buffer, pH 7.0 and 2 mL of 5% (w/v) potassium dichloromate and 1:3 (v/v) glacial acetic acid. The absorbance at 570 nm was read after 15 min using a Genesys 5 spectrophotometer.

3.2.8 Expression analysis by semi-quantitative RT-PCR

Frozen pericarp tissues were ground into a fine powder and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with additional phenol/chloroform extractions followed by RNA precipitation with isopropanol in the presence of Na citrate/NaCl. Total RNA was treated with RNase free DNase (Ambion, Austin, TX).

RNA was reverse transcribed into cDNA using random hexamers and a Retroscript kit (Ambion, Austin, TX) according to the manufacturer's instructions. Relative RT-PCR was performed with sets of gene specific primers and the universal 18S internal standards (Ambion, Austin, TX) as an internal control. PCR reactions were performed using Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), as outlined in Table 3.1. Amplification was done in triplicates using an Eppendorf Mastercycler (Hamburg, Germany) under the following conditions: initial denaturation at 94 ^{o}C for 2 min, followed by 27-30 cycles of denaturation at 94 ^{o}C for 45 sec, annealing for 45 sec, and extension at 72 ^{o}C for 45 sec. The final extension step was performed at 72 °C for 15 min. PCR products

Table 3.1 PCR primer sequences and PCR conditions used for the expression analysis of GABA shunt and antioxidant genes.

SGN Unigene ID	Primer	Primer sequence (5'-3')	Annealing temperature (^{o}C)	Number of cycles
SGN-U213860	GAD1_F	GAAAAGAAAAGCAGAAGGCAAA	50	27
	GAD1_R	CATACTTGTGACCGCTGACATT		
SGN-U212562	GAD2_F	CAAAAATTATGTTGACATGGATGAA	50	30
	GAD2_R	TTAAGAGTTGAACCCAAAATAGCAG		
SGN-U213635	GABAT_F	TCTGATGTAATTCATTCTCAAAGCA	50	30
	GABAT_R	TCCCATAGATTCTAATCAACTCGTC		
SGN-U220078	SSADH_F	CTCGGTTGAAACTAGTCCTTCATTA	50	30
	SSADH_R	CTAAAGCTGTCCAAAACATGAAAGT		
SGN-U214601	SSR_F	GGAGGAAATAGGGTTTCTAGGG	50	30
	SSR_R	CTTCTGCAGGAGTTTCTCCAAC		
SGN-U215177	APX_F	TGCTGAGATTAGCGTAAACTTTCTT	58	30
	APX_R	TTTCCAGATTTCCTAGTAACCACAA		
SGN-U223576	CAT1_F	TTTTACACCAGAGAGGGTAACTTTG	58	30
	CAT1_R	CAGCAATTGAATCATAGAGATCCTT		
SGN-U313439	CAT2_F	AGTTTTACACCAGAGAGGGAAACTT	58	30
	CAT2_R	ATTTCCAGCTGCAATAGAGTCATAG		
SGN-U215385	POX1_F	TGGATCAGTACTGTTAGATGGTTCA	55	30
	POX1_R	AGGGTAAAGTCTGTCTGTGAAAGAA		
SGN-U215392	POX2_F	AACTCAGCCAGAGGTTATGAAGTAA	55	30
	POX2_R	TAGTATCCAATTGTTGCAAATCAGA		
SGN-U215795	SOD_F	AATGTTCGTATAACTGGACTTGCTC	58	30
	SOD_R	CATTATAATTCCGACAAAAGAGCTG		

SGN-U= Solanaceae Genomics Network Unigene number.

were separated on 1.5% agarose gels containing ethidium bromide and the intensity of PCR products was measured by Quantity One software (Version 4.6.2, BioRad, Hercules, CA). The ratio between the intensity of the gene specific fragment and that of the 18S internal control band was calculated. The relative expression of each gene was an average of 3 independent replicates.

3.2.9 Statistical analysis

Statistical analysis of the chilling sensitivity screening data and chilling symptoms was conducted by one-way ANOVA and Duncan's multiple range tests of the Statistical Analysis System version 8.2 (SAS Institute, Cary, NC). Statistical analysis of all other data were subjected to analysis of variance using the General Linear Model (GLM) of the MINITAB software release 15 (Minitab Inc., State College, PA).

3.3 RESULTS

3.3.1 Chilling sensitivity screening of tomato introgression lines

Tomato lines used in this experiment were composed of the cultivated species, *S. lycopersicum* CV-M82, carrying introgressed genomic regions from the wild species *S. pennellii*. Exposure of fruit to 3 ^{o}C for 21 d caused surface pitting and secondary fungal decay in more chilling sensitive lines (Appendix). After cold storage plus 1 d at 20 ^{o}C, IL 3-4, IL 7-4-1, and IL 8-2-1 were the most chilling sensitive lines in that they showed the highest score of surface pitting (3.3, 2.8, and 2.5, respectively), while IL 8-1-2, IL 2-4, IL 11-2, IL 7-3, and IL 2-6 were the most

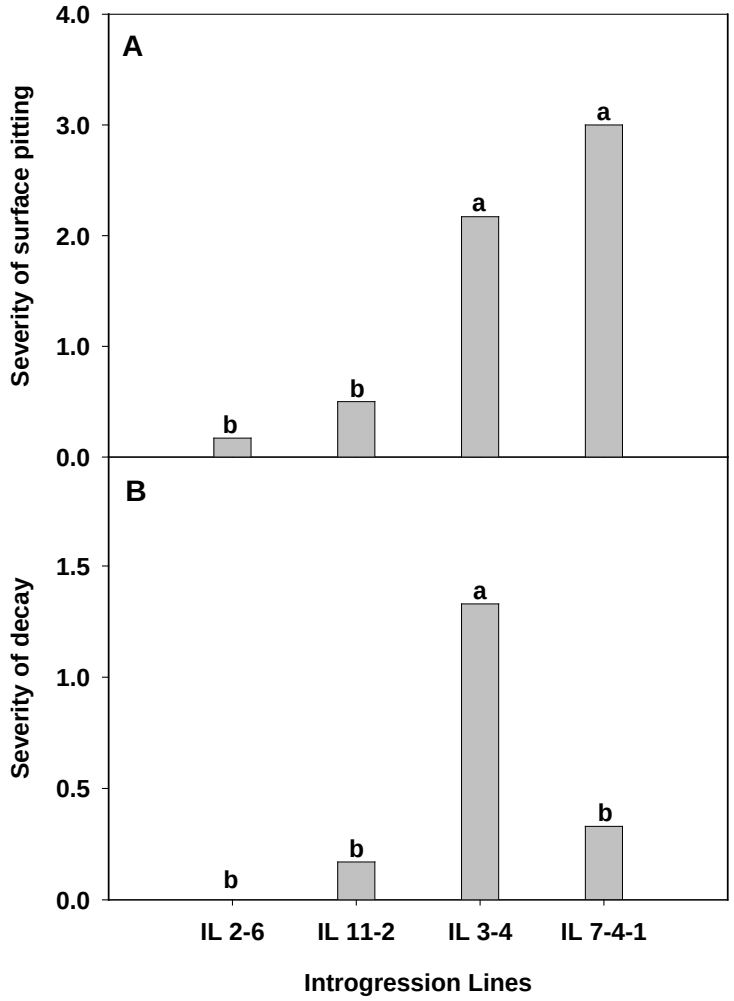


Figure 3.1 Surface pitting (A) and decay (B) incidence in tomato introgression lines stored for 28 d at 3 ^{o}C plus 14 d at 20 ^{o}C. Means with the same letter do not differ significantly at P= 0.05.

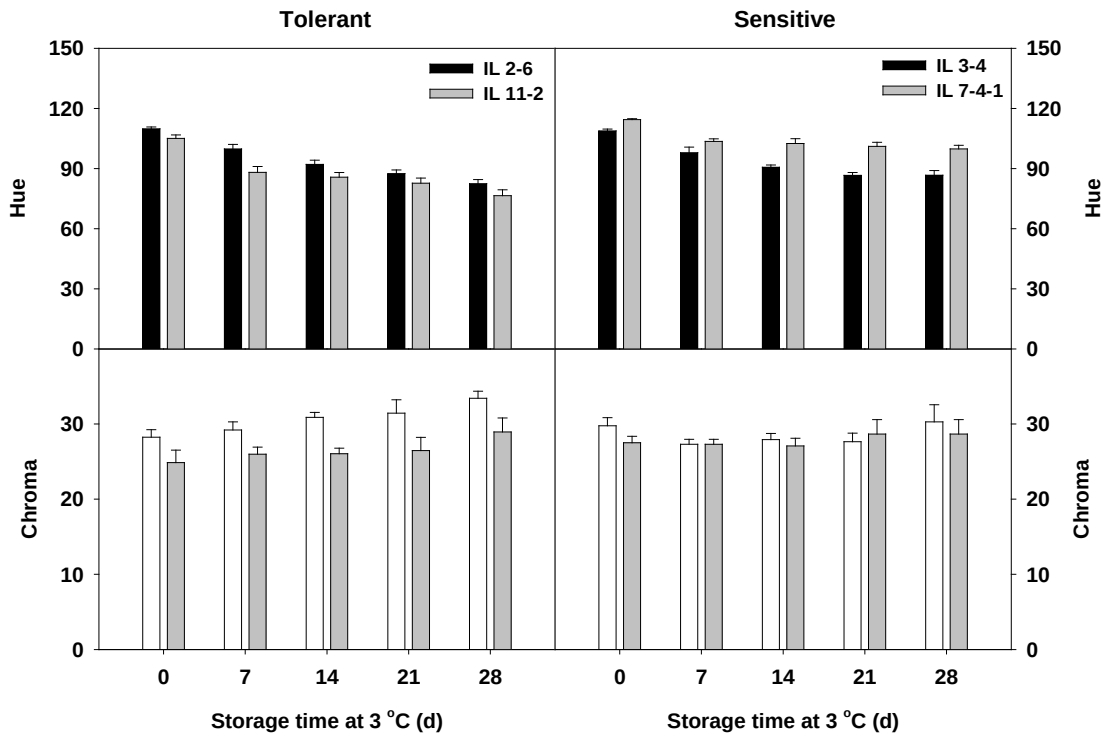
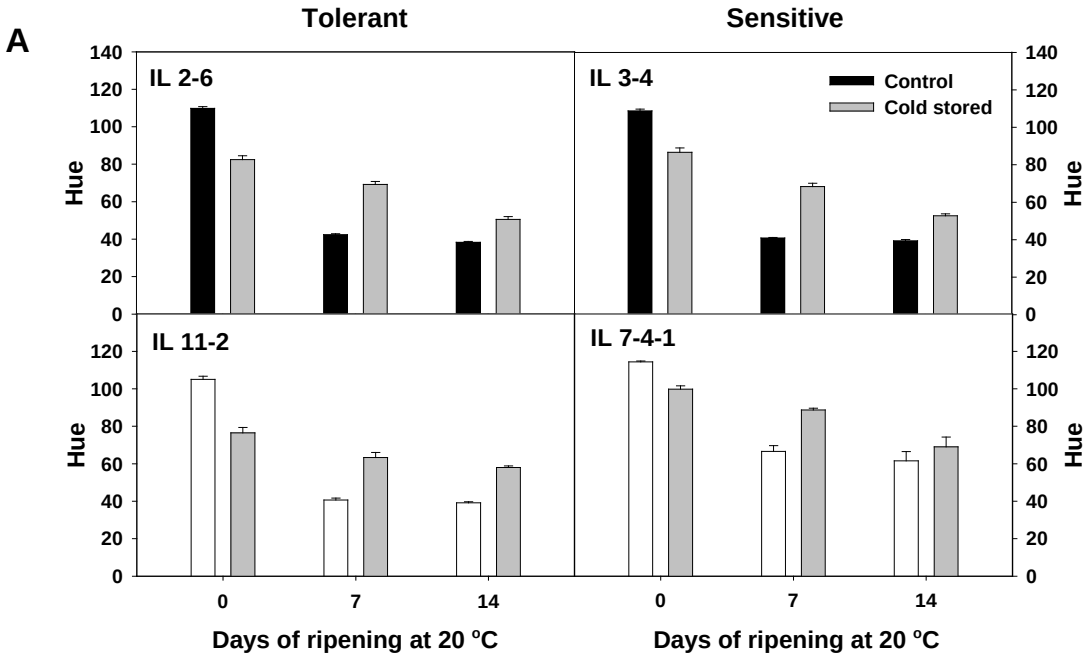
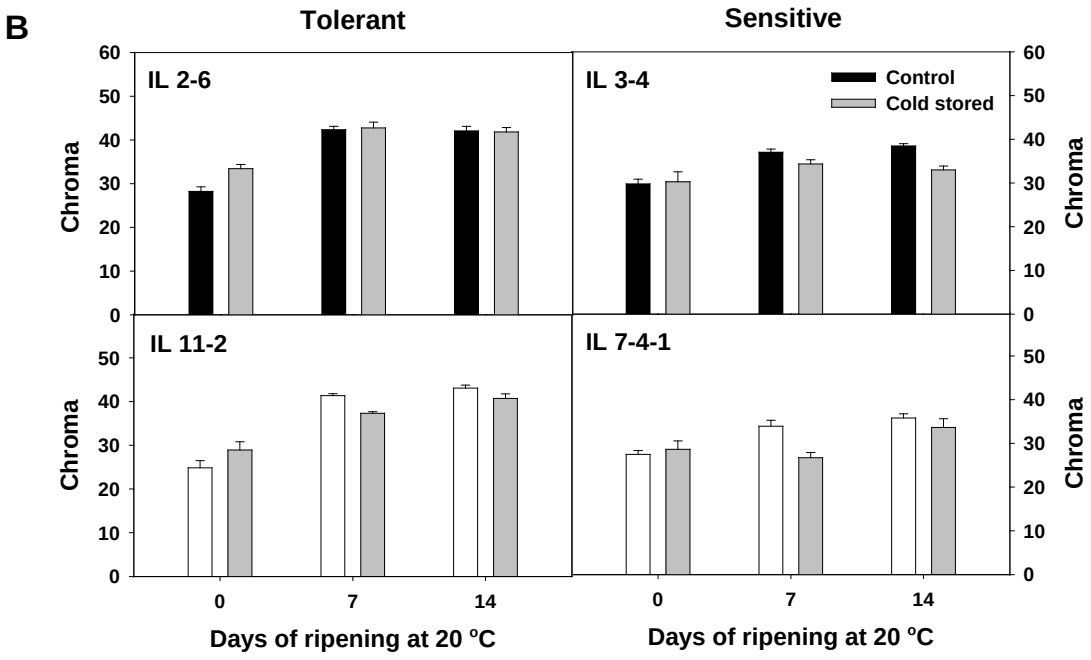


Figure 3.2 Hue angles and chroma values in tomato introgression lines during cold storage at 3 °C, presented as mean ± the standard error of the mean. For hue angles, line (l) and day (d) were significant at $P < 0.001$, while l x d was significant at $P = 0.02$. For chroma values, l was significant at $P < 0.001$, while d was significant at $P = 0.013$. No significant l x d interaction was found.

Figure 3.3 Hue angles (A) and chroma values (B) in tomato introgression lines during fruit ripening at 20 ^{o}C, presented as mean ± the standard error of the mean. Control fruit were not stored, while cold stored fruit were kept at 3 ^{o}C for 28 d prior to ripening. For Hue angles, line (l), day (d), storage (s), l x d, d x s were significant at $P < 0.001$, while l x d x s was significant at $P = 0.002$. For chroma values, l, d, l x d, and d x s were significant at $P < 0.001$, while s and l x s were significant at $P = 0.034$ and 0.006, respectively.


A
Tolerant
Sensitive
IL 2-6
IL 3-4
IL 11-2
IL 7-4-1
Control
Cold stored
Hue
0
20
40
60
80
100
120
140
0
7
14
Days of ripening at 20 °C


B
Tolerant
Sensitive
IL 2-6
IL 3-4
IL 11-2
IL 7-4-1
Control
Cold stored
Chroma
0
10
20
30
40
50
60
0
7
14
Days of ripening at 20 °C

chilling tolerant lines, with no sign of surface pitting. Secondary decay was detected after fruit were left at 20 ^{o}C for 7 d. Decay symptoms were more severe in fruit of IL 7-4-1, IL 8-1, IL 4-3, and IL 3-4 with the score greater than 2. There was no decay incidence in fruit of IL 2-4, IL 5-4, and IL 3-3. Based on these results, IL 3-4 and IL 7-4-1 were chosen as chilling sensitive lines, while IL 2-6 and IL 11-2 were chosen as chilling tolerant lines.

3.3.2 Comparison of chilling sensitive and tolerant lines

3.3.2.1 Fruit quality and chilling symptoms

In the greenhouse-grown tomatoes, CI symptoms of surface pitting and decay took longer to appear and the storage period was extended to 28 d. The incidence of surface pitting was severe in cold stored fruit of IL 3-4 and IL 7-4-1, and the average scores of surface pitting at 14 d after removal to 20 ^{o}C were 2.2 and 3.0, respectively. Cold stored fruit of IL 2-6 and IL 11-2 had little surface pitting. The highest decay incidence was in cold stored fruit of IL 3-4. The severity of decay was much less in fruit of IL 2-6, IL 11-2, and IL 7-4-1 and there was no significant difference in decay incidence in these 3 lines (Figure 3.1).

A decrease in hue angle during cold storage was observed in all lines (Figure 3.2). Color intensity (chroma) increased during cold storage but the increase was greater in tolerant lines (18% and 16% in IL 2-6 and IL 11-2, respectively) compared with sensitive lines (2% and 4% in IL 3-4 and IL 7-4-1, respectively). At 20 ^{o}C, chroma increased as fruit ripened, but the increase was lower in cold stored fruit of sensitive lines (Figure 3.3).

At day 14 of ripening at 20 ^{o}C, chroma values in fruit of sensitive lines were lower compared with control fruit by 13% and 14% in IL 3-4 and IL 7-4-1, respectively. The overall hue angle value decreased as fruit became redder during

ripening without storage. On day 14 of ripening after cold storage, fruit had 26%, 42%, 30% and 32% higher hue angle values in IL 2-6, IL 11-2, IL 3-4, and IL 7-4-1, respectively, compared with non-stored fruit.

3.3.2.2 Changes of GABA and GABA shunt enzymes during cold storage

GABA concentrations in fruit of the tolerant and sensitive lines were variable at harvest, with no consistent patterns that could be associated with susceptibility to CI (Figure 3.4). In the tolerant fruit, however, the GABA concentrations decreased 25% and 7% in IL 2-6 and IL 11-2, respectively, during cold storage. In contrast, the GABA concentrations in sensitive lines increased slightly (27% and 46% in IL 3-4 and IL 7-4-1, respectively) during cold storage. Thus, GABA concentrations in tolerant lines were lower after cold storage than at harvest, and in contrast, higher in sensitive lines (see also Figure 3.5).

No distinct patterns were observed for GAD activity between tolerant and sensitive lines during storage (Figure 3.4). GAD activity remained relatively unchanged during cold storage of IL 11-2, while activity of IL 2-6 decreased after day 7 and by 74% at the end of the storage period. In sensitive lines, a decrease of 54% in the enzyme activity was observed during storage of IL 7-4-1, whereas increased GAD activity was found in IL 3-4 over the first 14 d of storage before declining.

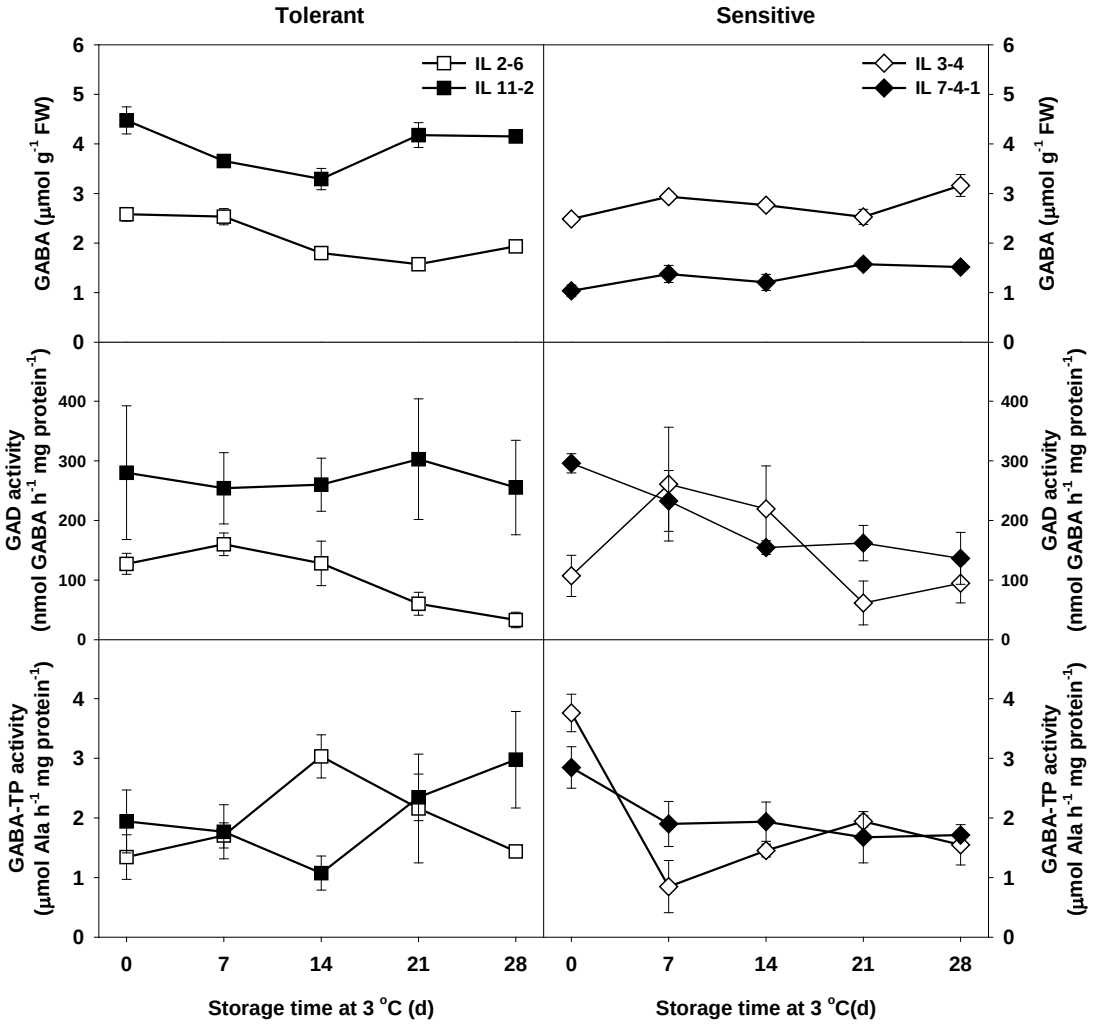


Figure 3.4 GABA concentrations, GAD activity, and GABA-T activity in tomato introgression lines during cold storage at 3 °C. Standard error values are indicated by vertical bars when bigger than symbols. For GABA, line (l) was significant at P <0.001, while day (d) was significant at P = 0.001. Interaction of l x d was significant at P <0.001. For GAD activity, l was significant at P <0.001. For GABA-T activity, the l x d interaction was significant at P <0.001.

Figure 3.5 GABA concentrations, GAD activity, and GABA-T activity in tomato introgression lines during fruit ripening, without cold storage or with storage at 3 ^{o}C for 28 d prior to ripening at 20 ^{o}C. Standard error values are indicated by vertical bars and are shown when bigger than symbols. For GABA, differences in line (l) and day (d) were significant at P < 0.001, and in l x d and line x storage (s) at $P = 0.001$. For GAD activity, d and s were significant at $P < 0.001$, while l and l x d were significant at 0.001 and 0.032, respectively. For GABA-T activity, l, and d were significant at $P < 0.001$, while s was significant at 0.044. Interactions of l x d, l x s, and d x s were significant at $P < 0.001$.

IL 2-6
IL 11-2
IL 3-4
IL 7-4-1
Control
Cold stored
GABA
(µmol g-1 FW)
GAD activity
(nmol GABA h-1 mg-1protein)
GABA-T activity
(µmol Ala h-1 mg-1protein)
Days of ripening at 20 °C

GABA-T activity was lower in both tolerant lines than in the sensitive lines at harvest (Figure 3.4). During cold storage, GABA-T activity fluctuated in the tolerant lines, but after 28 d had increased by 53% in IL 11-2. Activity in IL 2-6 increased to 126% of that in fruit at harvest at 14 d before declining. GABA-T activity decreased 59% in IL 3-4 and 40% in IL 7-4-1 during cold storage.

3.3.2.3. Changes of GABA and GABA shunt enzymes during fruit ripening

GABA concentrations were lower and higher in stored fruit of tolerant and sensitive lines, respectively, than at harvest (Figure 3.5). Although the concentrations declined during ripening of both control and cold stored fruit of all lines, the differences observed at harvest remained similar in trends, but the magnitude of these differences varied over time.

GAD activity decreased as fruit ripened (Figure 3.5). The enzyme activity was higher in control fruit of all lines compared with cold stored fruit, but the relative decrease in stored fruit was much greater in tolerant than sensitive fruit.

Overall GABA-T activity increased over time, but it was higher in cold stored than the control fruit of the tolerant lines (Figure 3.5). In contrast, the sensitive lines had lower or similar GABA-T activity in cold stored than control fruit.

3.3.2.4 Gene expression of GABA shunt enzymes

Relative expression of *GAD1* was much lower in cold stored fruit compared with fruit at harvest, but the expression increased in these fruit during subsequent ripening (Figure 3.6). In contrast, relative *GAD2* expression in cold stored fruit of IL 2-6 and IL 3-4 was similar to those of control fruit at harvest. The expression in stored fruit was higher in IL 11-2, but lower in IL 7-4-1 than fruit at harvest. The relative expression decreased as fruit ripened in both treatments of IL 2-6, IL 3-4, and IL 7-4-1. The expression increased in both treatments in IL11-2 during the first 7 d.

Expression then decreased in control fruit while it continued to increase in cold stored fruit.

Relative *GABA-T* expression in cold stored fruit of tolerant lines was higher or similar to those of untreated fruit (Figure 3.6). In contrast, the relative gene expression in cold stored fruit of sensitive lines decreased during fruit ripening.

Overall, relative expression of the *SSADH* gene was much higher in tolerant lines compared with sensitive lines (Figure 3.6). The gene expression remained unchanged in stored fruit of IL 11-2, but increased in stored fruit of other lines. The expression in control fruit of IL 3-4 increased over time, but decreased in other lines. The relative gene expression was greater in cold stored fruit compared with control fruit by the end of the ripening period.

The overall gene expression of succinic semialdehyde reductase (*SSR*) was much greater in the tolerant lines compared with sensitive lines (Figure 3.6). In tolerant lines, relative expression decreased over time during fruit ripening in both treatments. In sensitive lines, gene expression in stored fruit remained relatively constant during ripening. The expression slightly increased in control fruit of IL 3-4, but decreased in control fruit of IL 7-4-1.

Figure 3.6 Relative expression of the GABA shunt genes, *GAD1, GAD2*, *GABA-T*, *SSADH*, and *SSR*, in tomato introgression lines during fruit ripening, without cold storage or with storage at 3 ^{o}C for 28 d prior to ripening at 20 ^{o}C. For *GAD1*, line (l), day (d), storage (s), l x d, d x s, and l x d x s were significant at $P < 0.001$. For *GAD2*, l, d, l x d, l x d x s were significant at $P < 0.001$, while d x s was significant at $P = 0.002$. For *GABA-T*, l, d, l x s, d x s, l x d x s were significant at $P < 0.001$, while l x d was significant at $P = 0.001$. For *SSADH*, l, s, l x d, d x s, l x d x s were significant at $P < 0.001$, while d and l x s were significant at $P = 0.007$ and 0.001, respectively. For *SSR*, l, d, l x d, l x s were significant at $P < 0.001$, while d x s and l x d x s were significant at $P = 0.001$ and 0.003, respectively.

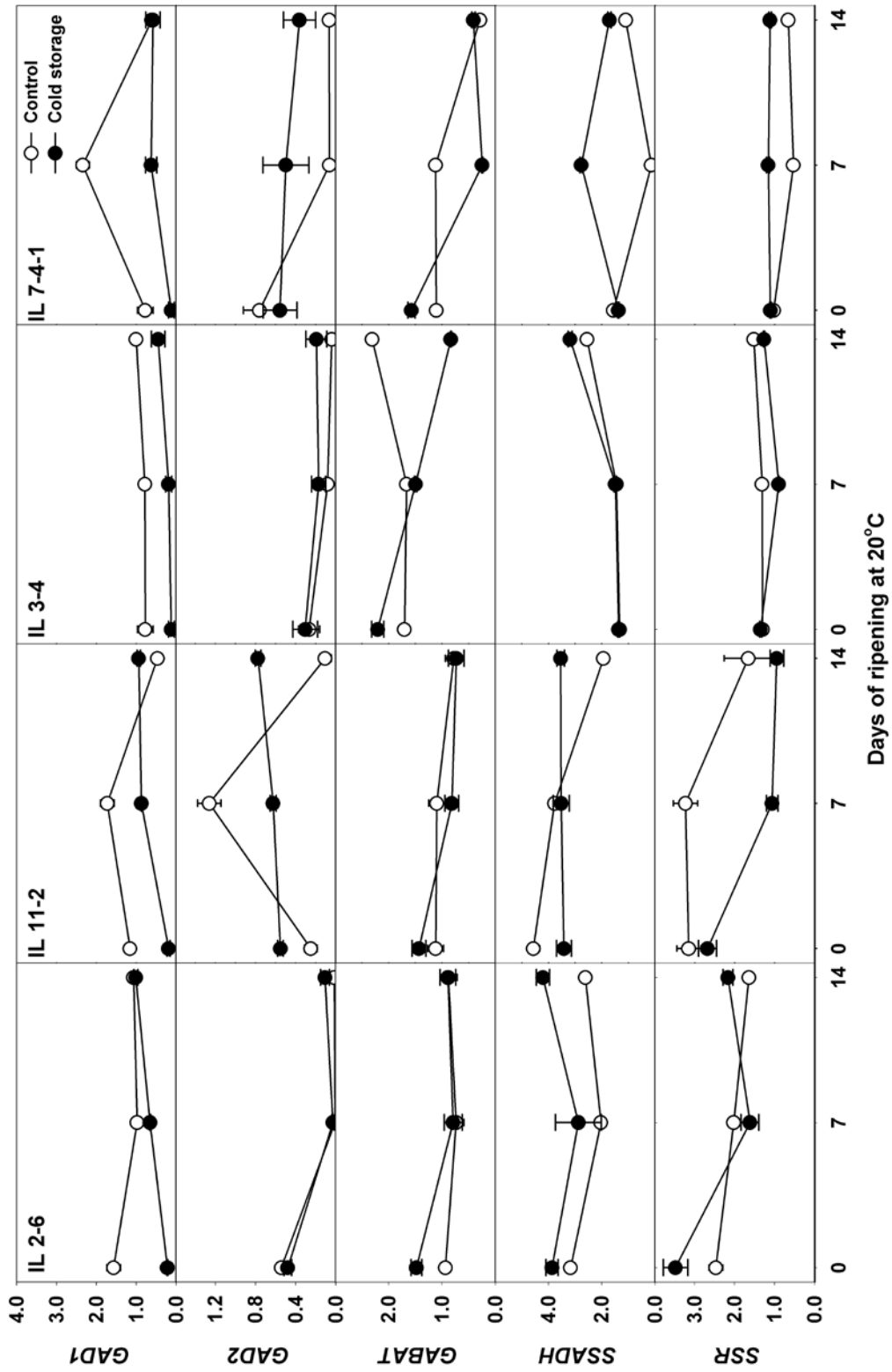

IL 2-6
IL 11-2
IL 3-4
IL 7-4-1
Control
Cold storage
GAD1
GAD2
GABAT
SSADH
SSR
Days of ripening at 20°C

Figure 3.7 Hydrogen peroxide (H_2O_2) levels, APX activity, CAT activity, POX activity, and SOD activity in tomato introgression lines during fruit ripening, without cold storage or with storage at 3 ^{o}C for 28 d prior to ripening at 20 ^{o}C. Standard error values are indicated by vertical bars when bigger than symbols. For H_2O_2, differences in line (l), day (d), l x d, d x storage (s), and l x d x s were significant at $P < 0.001$, while s and l x s were significant at $P = 0.026$ and 0.002, respectively. For APX, s, was significant at $P < 0.001$, while l x s was significant at $P = 0.006$. For CAT, d and s were significant at $P < 0.001$, while l x s was significant at $P = 0.006$. For POX, l, s, l x d, l x d x s were significant at $P < 0.001$, while l x s was significant at $P = 0.015$. For SOD, d, s were significant at $P < 0.001$, while d x s was significant at $P = 0.042$.

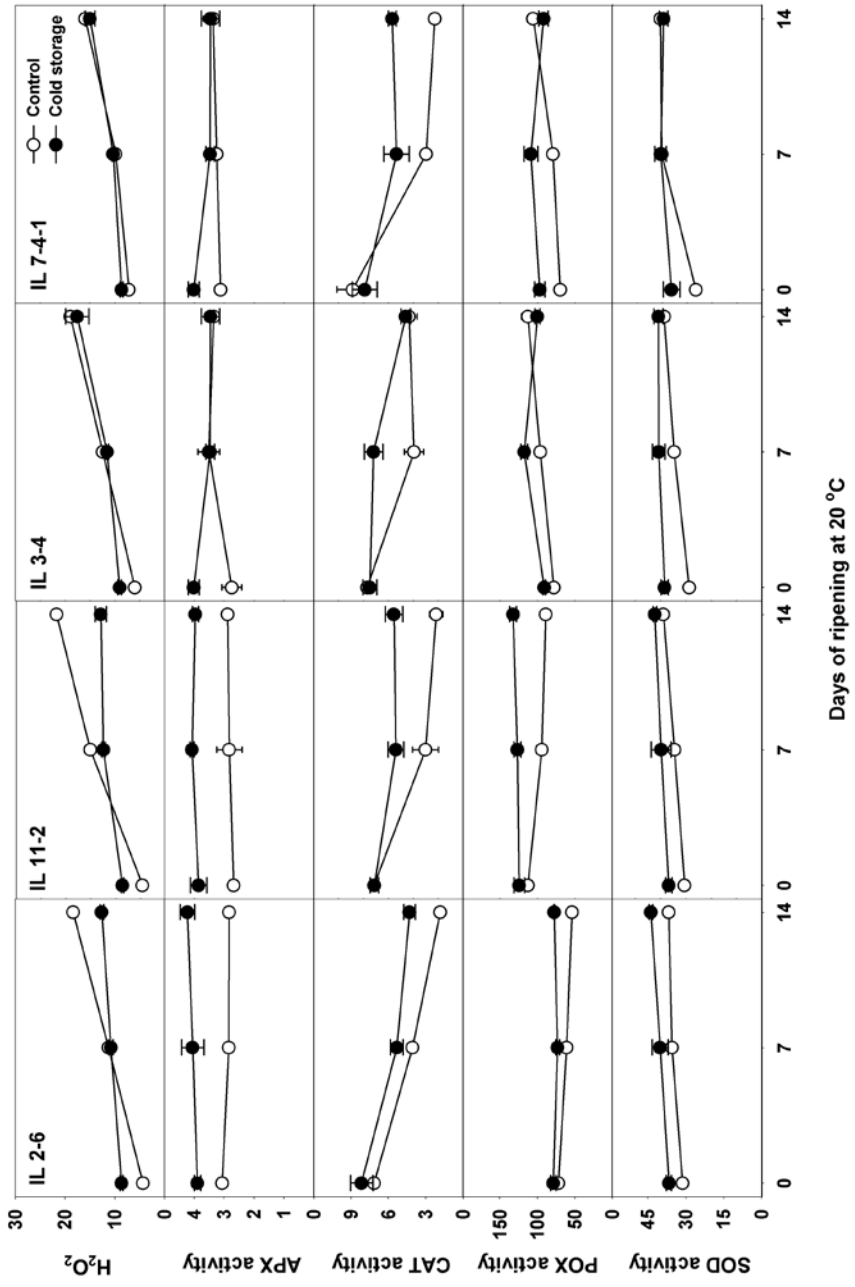
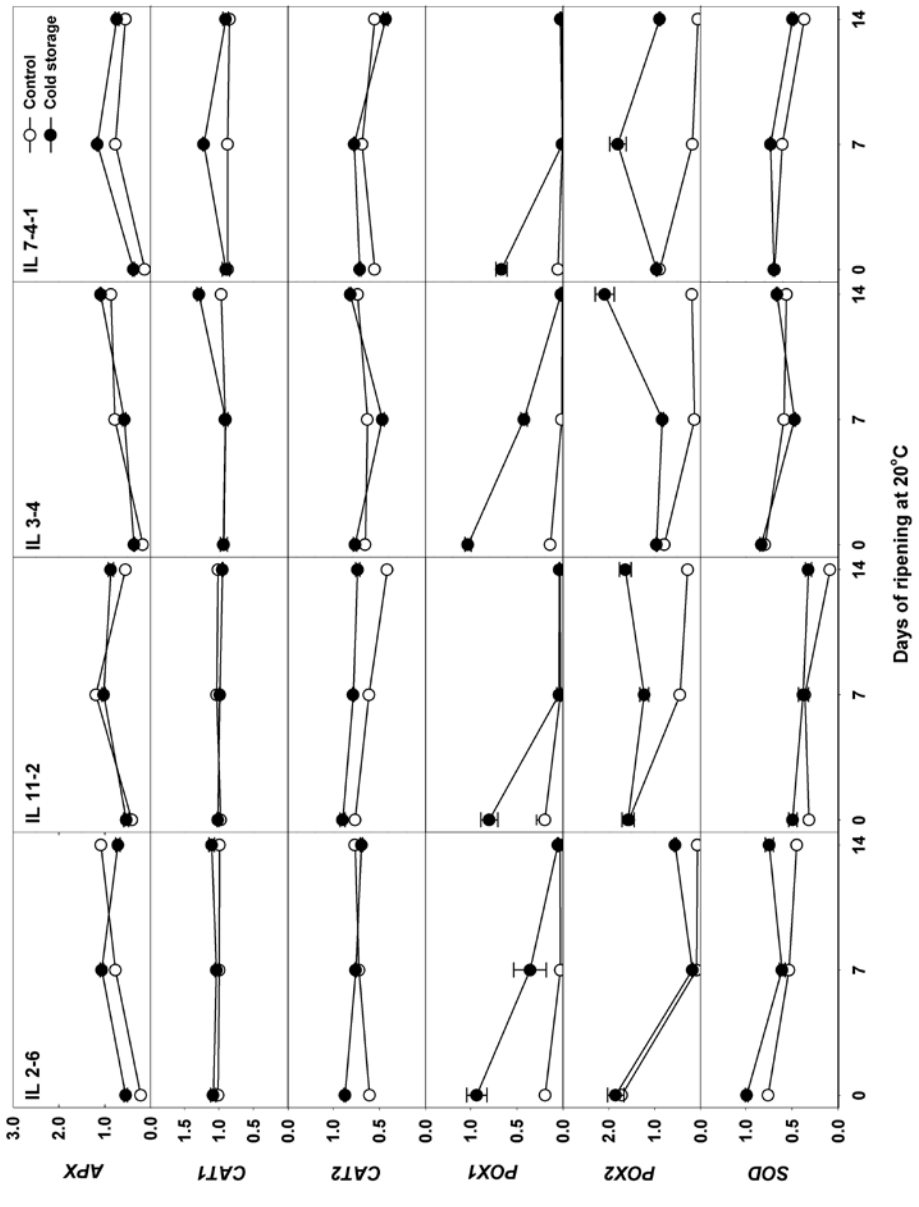

IL 2-6
IL 11-2
IL 3-4
IL 7-4-1
Control
Cold storage
H2O2
APX activity
CAT activity
POX activity
SOD activity
Days of ripening at 20 °C

Figure 3.8 Relative expression of the antioxidant enzyme genes, *APX*, *CAT1*, *CAT2*, *POX1*, *POX2*, and *SOD*, in tomato introgression lines during fruit ripening, without cold storage or with storage at 3 ^{o}C for 28 d prior to ripening at 20 ^{o}C. For *APX*, line (l), day (d), storage (s), l x d, l x s, l x d x s were significant at $P < 0.001$, while d x s was significant at $P = 0.001$. For *CAT1*, l, s, l x d, l x d x s were significant at $P < 0.001$, while l x s was significant at $P = 0.002$. For *CAT2*, all factors and interactions were significant at $P < 0.001$. For *POX1*, l, d, s, d x s were significant at $P < 0.001$, while l x d was significant at $P = 0.022$. For *POX2*, all factors and interactions were significant at $P < 0.001$. For SOD, l, d, s, l x d, l x s, and d x s were significant at $P < 0.001$, while l x d x s was significant at $P = 0.014$.


IL 2-6
IL 11-2
IL 3-4
IL 7-4-1
Control
Cold storage
APX
CAT1
CAT2
POX1
POX2
SOD
Days of ripening at 20°C

3.3.2.5 Hydrogen peroxide content

The H_2O_2 concentrations were higher in cold stored fruit of all lines before transfer to warmer temperature than the control fruit without cold storage (Figure 3.7). In the tolerant fruit, the H_2O_2 concentrations in the stored fruit then increased during ripening, but at a slower rate than the control fruit. However, little effect of cold storage on the H_2O_2 increase during ripening of the sensitive fruit lines was evident.

3.3.2.6 Antioxidant enzymes

Cold stored fruit before transfer to 20 ^{o}C had greater APX activities compared with fruit at harvest in all lines (Figure 3.7), but activity remained higher in stored fruit of only tolerant lines during ripening.

There were no differences in CAT activity between cold stored fruit and control fruit at harvest (Figure 3.7). The activity in both non-treated and cold stored fruit declined during fruit ripening, but the activity was higher in stored fruit of all lines.

POX activity was greater in cold stored fruit than in fruit at harvest time (Figure 3.7). In tolerant lines, the activity decreased in control fruit, but remained relatively stable in stored fruit. In sensitive lines, the activity increased in stored fruit but then decreased after day 7, while the activity continued to increase in control fruit. By the end of the ripening period, the activity in stored fruit was similar to those of control fruit.

Cold stored fruit showed a higher SOD activity compared with fruit at harvest (Figure 3.7). The overall enzyme activity increased during ripening, but was not affected by line.

3.3.2.7 Gene expression of antioxidant enzymes

APX gene expression in cold stored fruit was greater than in control fruit at harvest (Figure 3.8). The relative expression increased during fruit ripening, but

different patterns of change appeared within both the tolerant and the sensitive lines, and there was no clear distinction between the two groups.

CAT1 relative expression in cold treated fruit was similar to that in fruit at harvest (Figure 3.8). Overall, gene expression was stable over time. *CAT2* expression was higher in cold treated fruit compared with fruit at harvest. The gene expression in stored fruit decreased as fruit ripened except for IL 3-4 in which an increase following a decrease was detected. However, no clear gene expression patterns of *CAT1* and *CAT2* between tolerant and sensitive lines were observed.

The relative expression of *POX1* in cold stored fruit was greater than in fruit at harvest (Figure 3.8). The expression declined over time as fruit ripened. The expression in cold stored fruit decreased to the same levels as those of non-stored fruit by the end of the ripening stage. The relative expression of *POX2* was greater in cold stored fruit than in control fruit. The expression decreased as fruit ripened. No clear separation in the expression of *POX1* and *POX2* between tolerant and sensitive lines was found.

Higher *SOD* expression in cold stored fruit was observed only in tolerant lines (Figure 8). The relative expression decreased during fruit ripening. The gene expression was greater in stored fruit in all lines by the end of the ripening period, but to a greater extent in tolerant lines.

3.4 DISCUSSION

The responses of two lines of tolerant and two lines of sensitive tomato fruit to cold storage have been compared. Clear differences in pitting and decay were detected between the two groups. All lines ripened after cold storage, but less rapidly than the non-stored lines as indicated by slower changes in hue and chroma values. However,

no consistent effects on the effect of cold storage on ripening inhibition between tolerant and sensitive lines were detected, despite a difference in susceptibility to CI.

GABA and glutamate are the predominant amino acids in tomato fruit. GABA accumulates in fruit during development and reaches its highest levels prior to the breaker stage and then decreases (Kisaka et al., 2006a; Akihiro et al., 2008a). In addition to changes during fruit development, GABA contents in tomato vary greatly depending on cultivar or genotype and its concentration ranges from 0.8 to 12 μmol g^{-1} FW in red stage tomato (Pratta et al., 2004; Saito et al., 2008). Chilling temperatures have been shown to result in GABA accumulation in plant tissues as diverse as barley, wheat, and cherimoya (Escribano and Merodio, 2001; Mazzucotelli et al., 2006), and increases vary from 3.6 times in cherimoya to 19 times in wheat. However, our study only showed slight GABA accumulation in tomato fruit of the sensitive lines (1.3 and 1.5 times in Il 3-4 and IL 7-4-1, respectively). GABA concentrations in tolerant lines were not affected or slightly lower when fruit were stored for up to 28 d at 3 ^{o}C. GABA concentrations decreased during ripening at 20 °C, whether or not fruit had been stored. This indicates that decreased synthesis and/or more rapid degradation of GABA in tomato fruit occurs throughout ripening, regardless of storage conditions.

No distinct differences in gene expression of *GAD1*, *GAD2*, and *GABA-T* were observed. However, while measurements of GABA shunt enzymes did not reveal induction of GAD activity by chilling stress, reduced GABA-T activity was found in cold stored fruit of sensitive lines during cold storage. GAD activity decreased during fruit ripening, while GABA-T activity increased as fruit ripened. These findings suggest that GABA-T may play important roles in controlling GABA levels in tomato fruit. Evidence that GAD plays an important role in regulating GABA and glutamate metabolism includes abnormal development in plants with down-regulation of a *GAD* gene (Baum et al., 1996a; Bouche et al., 2004b). However, fruit of antisense *GAD*

tomatoes showed elevated levels of glutamate, but no significant differences in GABA concentrations between transformants and the wild type (Kisaka et al., 2006a). These findings and our results suggest that GABA-T may play a more important role than GAD in regulating GABA concentration. GABA-T can metabolize GABA to SSA only if amino group receptors are available. On the other hand, SSA can be used to produce GABA in mitochondria by the reverse reaction of GABA-T when SSADH is impaired. It is possible that this bifunctional enzyme may serve as a regulator to maintain cellular GABA levels.

Interestingly, the overall mRNA levels of *SSADH* and *SSR* genes were much higher in tolerant lines than in the sensitive lines. This is consistent with results from a previous study in which frost resistant barley exhibited high levels of GABA-shunt genes during freezing (Mazzucotelli et al., 2006). A study in yeast showed that mutants lacking *GABA-T* and *SSDAH* were more sensitive to high temperature, while *GAD* mutants were very similar to the wild type, and over-expression of *Arabidopsis GABA-T* in yeast *GABA-T* mutants could restore the sensitivity to the same level as the wild type (2005). Therefore, the loss of the ability to maintain GABA catabolism in cold stored fruit of sensitive lines may have caused an increase in the net GABA accumulation, and suggests that the balance between biosynthesis and degradation of GABA may play a role in determining chilling sensitivity of tomato fruit.

It is believed that both chilling temperature and ripening are causes of oxidative stress and therefore it is assumed that fruit exposed to chilling temperatures prior to ripening must cope with oxidative stress from the fruit ripening process as well as from the chilling stress. Our results showed that H_2O_2 production was induced by chilling stress and H_2O_2 content increased during at 20 ^oC; however, the increase during fruit ripening in tolerant lines was lower in cold stored fruit than in the control

fruit suggesting differences in the antioxidant system between sensitive and tolerant lines.

The balance between SOD, APX and CAT activity is important in maintaining the steady-state level of superoxide radicals and H_2O_2 in plant cells (Mittler, 2002). In tomato fruit, H_2O_2 content increased during climacteric fruit ripening (Frenkel and Eskin, 1977; Warm and Laties, 1982), and the increases associated with decreased activities of SOD and POX (Rabinowich et al., 1982; Jimenez et al., 2002). After fruit were re-warmed to 20 ^{o}C, an increase in oxidative stress caused by chilling stress did not seem to have much effect on activity of antioxidant enzymes during fruit ripening in sensitive lines. In tolerant lines, APX activity was greater in cold stored fruit and remained high during fruit ripening, while POX activity increased in cold stored fruit only after fruit were re-warmed. This suggests that APX might be responsible for ROS scavenging during chilling stress and fruit ripening, while POX might be responsible for ROS scavenging during fruit ripening only. In contrast to other enzymes**,** CAT activity declined during fruit ripening, and it seemed to be insensitive to changes in H_2O_2 levels during cold storage and early stages of fruit ripening. A similar decrease in CAT activity during tomato ripening was reported by Malacrida *et al.* (2006). This finding can be explained by the fact that APX and CAT differ in their affinity for H_2O_2. APX has a much higher affinity for H_2O_2, while CAT has a high reaction rate but very low affinity (Mittler, 2002). In this case, the fact that no induction of CAT activity was observed may be due to the absence of sudden and dramatic change in H_2O_2 in the pericarp.

Mondal *et al.* (2004) previously reported that a tomato cultivar with a longer shelf-life showed higher activity of antioxidant enzymes during fruit ripening. In our studies, cold stored fruit of tolerant lines exhibited more efficient mechanisms to detoxify H_2O_2, resulting in lower levels of H_2O_2 at the end of the ripening period. It is

possible that additional oxidative stress caused by chilling stress and the post-chilling respiratory burst in fruit of tolerant lines caused changes in ROS-scavenging systems, leading to an increase in mobilization of antioxidant defense mechanisms. This assumption is supported by higher activity of APX and POX in cold stored fruit of the two tolerant lines throughout the ripening period. In addition, activities of SOD and CAT were higher in cold-stored fruit of the two tolerant lines at the end of the ripening period.

Cold storage also resulted in increased transcript levels of *APX*, *CAT2* and *POX1* in both tolerant and sensitive lines, while SOD expression was induced by chilling stress only in tolerant lines. Even though transcription of some of the antioxidant enzymes in sensitive lines was found at higher levels in cold-stored fruit, the decrease in activity of those enzymes would reduce the ability of cells to lower H_2O_2 levels. Our results suggest that chilling tolerant lines were able to balance the production and detoxification of ROS, while chilling sensitive lines failed to inactivate ROS produced during fruit ripening.

Several reports have shown a possible relationship between the GABA shunt and accumulation of ROS during abiotic stresses. *Arabidopsis ssadh* knockout mutants were sensitive to UV and heat stresses, causing a striking increase in H_2O_2 (Bouche et al., 2003a). The GABA shunt has been explained as a bypass of the tricarboxylic acid (TCA) cycle in the production of succinate but a reduction of succinic semialdehyde (SSA) to GHB is an alternative route when SSADH is impaired. GHB was found to accumulate in *Arabidopsis ssadh* knockout mutants, but treatment with an GABA-T inhibitor could prevent the accumulation of H_2O_2 and GHB (Fait et al., 2005b). Ludewig *et al.* (2008b) showed that H_2O_2 only accumulated in *ssadh* mutants but not in *pop2* and double *pop2 ssadh* mutants, and *pop2 ssadh* mutants were highly sensitive to exogenous SSA and GHB, suggesting accumulation of these two metabolites in the

mutants. Moreover, GABA accumulation was also found in both *pop2* and *pop2 ssadh* mutants, but unlike in *ssadh* mutants, its accumulation was not associated with high accumulation of peroxides.

Taken together, an increase in GABA levels in sensitive lines may be due to a reduction of GABA-T activity combined with SSADH activity associated with lower gene expression of *SSADH*. In addition, lower *SSADH* and *SSR* mRNA levels in sensitive lines may make cells accumulate more SSA and GHB, which seem to be toxic to plant cells, and this accumulation may in turn result in higher peroxide accumulation. Thus**,** maintaining the intact GABA shunt pathway is possibly a key to avoiding damage from accumulation of toxic GABA shunt metabolites and ROS.

REFERENCES

Akihiro, T., Koike, S., Tani, R., Tominaga, T., Watanabe, S., Iijima, Y., Aoki, K., Shibata, D., Ashihara, H., Matsukura, C., Akama, K., Fujimura, T., Ezura, H., 2008. Biochemical mechanism on GABA accumulation during fruit development in tomato. Plant Cell Physiol. 49, 1378-1389.

Ansari, M.I., Lee, R.H., Chen, S.C.G., 2005. A novel senescence-associated gene encoding γ-aminobutyric acid (GABA):pyruvate transaminase is upregulated during rice leaf senescence. Physiol. Plantarum 123, 1-8.

Apel, K., Hirt, H., 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Ann. Rev. Plant Biol. 55, 373-399.

Aurisano, N., Bertani, A., Reggiani, R., 1995. Involvement of calcium and calmodulin in protein and amino-acid metabolism in rice roots under anoxia. Plant Cell Physiol. 36, 1525-1529.

Bartyzel, I., Pelczar, K., Paszkowski, A., 2003. Functioning of the γ-aminobutyrate pathway in wheat seedlings affected by osmotic stress. Biol. Plantarum 47, 221-225.

Baum, G., Lev-Yadun, S., Fridmann, Y., Arazi, T., Katsnelson, H., Zik, M., Fromm, H., 1996. Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. EMBO J. 15, 2988-2996.

Boonsiri, K., Ketsa, S., Van Doorn, W.G., 2007. Seed browning of hot peppers during low temperature storage Postharvest Biol. Technol. 45, 358-365.

Bouche, N., Fait, A., Bouchez, D., Moller, S.G., Fromm, H., 2003. Mitochondrial succinic-semialdehyde dehydrogenase of the γ-aminobutyrate shunt is required

to restrict levels of reactive oxygen intermediates in plants. PNAS 100, 6843-6848.

Bouche, N., Fait, A., Zik, M., Fromm, H., 2004. The root-specific glutamate decarboxylase (GAD1) is essential for sustaining GABA levels in *Arabidopsis*. Plant Mol. Biol. 55, 315-325.

Cao, J., Barbosa, J.M., Singh, N.K., Locy, R.D., 2005. The role of the GABA shunt in thermotolerance., Plant Biology 2005. American Society of Plant Biologists, Seattle, Washington, USA.

Escribano, M.I., Merodio, C., 2001. Limited ammonium assimilation in cherimoya fruit stored at chilling temperature. Acta Hort. 533, 311-312.

Eshed, Y., Zamir, D., 1994. A genomic library of *Lycopersicon pennellii* in *L. esculentum*: A tool for fine mapping of genes. Euphytica 79, 175-179.

Fait, A., Yellin, A., Fromm, H., 2005. GABA shunt deficiencies and accumulation of reactive oxygen intermediates: insight from *Arabidopsis* mutants. FEBS Lett. 579, 415-420.

Foyer, C.H., Lelandais, M., Kunert, K.J., 1994. Photooxidative stress in plants. Physiol. Plant. 92, 696-717.

Franck, C., Lammertyna, J., Tri Ho, Q., Verboven, P., Verlinden, B., Nicola, B.M., 2007. Browning disorders in pear fruit. Postharvest Biol. Technol. 43, 1-13.

Frenkel, C., Eskin, M., 1977. Ethylene evolution as related to changes in hydroperoxides in ripening tomato fruit. HortSci. 12, 552-553.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases. Plant Physiol. 59, 309-314.

Hodges, D.M., Andrews, C.J., Johnson, D.A., Hamilton, R.I., 1997. Antioxidant enzyme and compound responses to chilling stress and their combining abilities in differentially sensitive maize hybrids. Crop Sci. 37, 857-863.

Hulme, A.C., Arthington, W., 1950. γ–Aminobutyric acid and β–alanine in plant tissue. Nature 165, 716-717.

Jimenez, A., Creissen, G., Kular, B., Firmin, J., Robinson, S., Verhoeyen, M., Mullineaux, P., 2002. Changes in oxidative processes and components of the antioxidant system during tomato fruit ripening. Planta 214, 751-758.

Kinnersley, A.M., Turano, F.J., 2000. Gamma aminobutyric acid (GABA) and plant responses to stress. Crit. Rev. Plant Sci. 19, 479-509.

Kisaka, H., Kida, T., Miwa, T., 2006. Antisense suppression of glutamate decarboxylase in tomato (*Lycopersicon esculentum* L.) results in accumulation of glutamate in transgenic tomato fruits. Plant Biotech. 23, 267-274.

Ludewig, F., Huser, A., Fromm, H., Beauclair, L., Bouche, N., 2008. Mutants of GABA transaminase (POP2) suppress the severe phenotype of succinic semialdehyde dehydrogenase (ssadh) mutants in *Arabidopsis*. PLoS ONE 3, e3383.

Makino, Y., Soga, N., Oshita, S., Kawagoe, Y., Tanaka, A., 2008. Stimulation of γ-aminobutyric acid production in vine-ripe tomato (*Lycopersicon esculentum* Mill.) fruits under modified atmospheres. J. Agri. Food Chem. 56, 7189-7193.

Malacrida, C., Valle, E.M., Boggio, S.B., 2006. Postharvest chilling induces oxidative stress response in the dwarf tomato cultivar Micro-Tom. Physiol. Plantarum 127, 10-18.

Mayer, R.R., Cherry, J.L., Rhodes, D., 1990. Effects of heat shock on amino acid metabolism of cowpea cells. Phytochemistry 94, 796-810.

Mazzucotelli, E., Tartari, A., Cattivelli, L., Forlani, G., 2006. Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. J. Exp. Bot. 57, 3755-3766.

Merodio, C., Munoz, M.T., Cura, B.D., Buitrago, D., Escribano, M.I., 1998. Effect of high CO_2 level on the titres of γ-aminobutyric acid, total polyamines and some pathogenesis-related proteins in cherimoya fruit stored at low temperature. J. Exp. Bot. 49, 1339-1347.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405-410.

Mondal, K., Sharma, N.S., Malhotra, S.P., Dhawan, K., Singh, R., 2004. Antioxidant systems in ripening tomato fruits. Biol. Plantarum 48, 49-53.

Nakano, L., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867-880.

Pedreschi, R., Franck, C., Lammerteyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123-130.

Pratta, G., Zorzoli, R., Boggio, S.B., Picardi, L.A., Valle, E.M., 2004. Glutamine and glutamate levels and related metabolizing enzymes in tomato fruits with different shelf-life. Sci. Hortic. 100, 341-347.

Rabinowich, H.D., Sklan, D., Budowski, P., 1982. Photo-oxidative damage in the ripening tomato fruit: protective role of superoxide dismutase. Physiol. Plantarum 54, 369-374.

Rao, M.V., Paliyath, G., Ormrod, D.P., 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol. 110, 125-136.

Robert, E., 1988. The establishment of GABA as a neurotransmitter. In: Squirs, R.F. (Ed.), GABA and Benzodiazepine Receptors. CRC Press, Boca Raton. pp. 1-21.

Saito, T., Matsukura, C., Sugiyama, M., Watahiki, A., Ohshima, I., Iijima, Y., Konishi, C., Fujii, T., Inai, S., Fukuda, N., Nishimura, S., Ezura, H., 2008. Screening for γ-aminobutyric acid (GABA)-rich tomato varieties. J. Japan. Soc. Hort. Sci. 77, 242-250.

Streeter, J.G., Thompson, J.F., 1972. Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). Plant Physiol. 49, 572-584.

Thompson, J.F., 2002. Storage systems. In: Kader, A.A. (Ed.), Postharvest Technology of Horticultural Crops. University of California Agriculture and Natural Resources, California. pp. 535.

Tsushida, T., Murai, T., 1987. Conservation of glutamic acid to γ-aminobutyric acid in tea leaves under anaerobic conditions. Agric. Biol. Chem. 51, 2865-2871.

Wallace, W., Secor, J., Schrader, L.E., 1984. Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol. 75, 170-175.

Warm, E., Laties, G.G., 1982. Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. Phytochemistry 21, 827-831.

Zhang, G., Bown, A.W., 1997. The rapid determination of γ-aminobutyric acid. Phytochemistry 44, 1007-1009.

Zushi, K., Matsuzoe, N., 2007. Salt stress-enhanced γ-aminobutyric acid (GABA) in tomato fruit. Acta Hort. 761, 431-435.

CHAPTER 4

γ- AMINOBUTYRIC ACID METABOLISM IN CO_2 TREATED TOMATOES

ABSTRACT

γ- Aminobutyric acid (GABA) metabolism has been investigated in breaker and light red maturation stages of 'Tricia' tomatoes exposed to air, or 10% CO_2 in air during storage, at 13 ^{o}C for 12 d, to examine whether GABA responses to elevated CO_2 differ in fruit at different maturity stages. At harvest, the GABA concentration in red fruit was about a third of that in breaker fruit. The ripening of breaker stage fruit was inhibited by CO_2 to a greater extent than the red fruit as judged by hue and chroma values, but the red fruit were more sensitive to development of water soaking, pitting and decay than the breaker fruit. During storage, GABA concentrations in breaker fruit decreased during storage but were usually higher in CO_2 treated than air treated fruit. GABA concentrations in red fruit increased in CO_2 treated fruit, but they increased and then decreased in air treated fruit over time. When CO_2 treated fruit were transferred to air, GABA concentrations declined to similar levels as those found in fruit stored in air. Although glutamate decarboxylase (GAD) activity was higher in breaker than red fruit at harvest, activities in air and CO_2 treated fruit were similar. GABA transaminase (GABA-T) activity was higher in red than breaker fruit, but activity declined more in CO_2 than air treated fruit. Although, CO_2 treatment did not affect *GAD1* expression, it resulted in higher *GAD2* and *GAD3* expression, and to a greater extent in breaker than red fruit. No consistent effects of high CO_2 conditions on the expression of *GABA-T* or succinic semialdehyde dehydrogenase (*SSADH*) genes were found. A decline in succinic semialdehyde reductase 1 (*SSR1*) gene expression was found only in CO_2 treated red fruit, while CO_2 treatment resulted in a

more rapid decrease of *SSR2* expression in both breaker and red fruit. The results reveal differences in GABA metabolism in breaker and red fruit in response to CO_2 treatment.

4.1 INTRODUCTION

γ-Aminobutyric acid (GABA) is a four-carbon non-protein amino acid that is found in animals, plants and bacteria. GABA is metabolized via a pathway called the GABA shunt that consists of three enzymes: glutamate decarboxylase (GAD), which catalyzes the conversion of glutamate to GABA; GABA transaminase (GABA-T), which catalyzes transamination conversion of GABA to succinic semialdehyde (SSA); and succinic semialdehyde dehydrogenase (SSADH), which catalyzes the irreversible NADP-dependent oxidation of SSA to succinate. Alternatively, SSA can be reduced to γ-hydroxybutyric acid (GHB) by succinic semialdehyde reductase (SSR) (Shelp et al., 1999; Allan et al., 2003; Breitkreuz et al., 2003b; Fait et al., 2005a; Allan et al., 2008b). GABA receptors are found in animals but not plants, although GABA may interact with plant glutamate receptors (Lancien and Roberts, 2006).

In animals, GABA is known to function as an important inhibitory neurotransmitter (Bowery and Smart, 2006) and may have antihypersensitivity, natriuretic and other positive effects (Antonaccio and Taylor, 1977; Abe et al., 1995; Yamakoshi et al., 2007; Shimada et al., 2009). In Japan there has been special interest in GABA as a health related compound, and the tomato has been identified as a good source of dietary GABA (Matumoto et al., 1997; Saito et al., 2008). A GABA-rich tomato cultivar was shown to reduce systolic blood pressure in spontaneously hypersensitive rats (Yoshimura et al., 2010).

The functional significance of GABA in plants, however, is still not well understood. Multiple roles for GABA have been proposed for plants, including

signaling, cell guidance, defense against insects, pH regulation, redox regulation, energy balance, stress responses, and in carbon and nitrogen metabolism (Satya Narayan and Nair, 1990b; Kinnersley and Turano, 2000; Bouche et al., 2003c; Bouche and Fromm, 2004a; Fait et al., 2005a). A role for GABA in mediating stress responses has been suggested because of its accumulation in plant tissues after exposure to a range of stresses including acidiosis, mechanical damage, salinity, heat, cold and drought (Kinnersley and Turano, 2000); however, GABA may play a major role in carbon and nitrogen metabolism and be an integral part of the TCA cycle under both stress and non-stress conditions (Fait et al., 2007b).

Little information about GABA metabolism in horticultural products after harvest is available. A study in mature green tomato fruit treated with 20% CO_2 for 3 d showed a sharp increase in expression of a ripening-regulated heat shock protein and GAD genes, but the expression strongly decreased when fruit were transferred to air (Rothan et al., 1997). This finding suggests a possible link between GABA and high CO_2 stress in tomato. GABA accumulated in tomatoes exposed to 11% O_2 and 9% CO_2 at 30 ^oC (Makino et al., 2008). GABA accumulation was found in cherimoya fruit treated with 20% CO_2 (Merodio et al., 1998a), in pears stored in 1% O_2 and 10% CO_2 where there was a higher incidence of flesh browning disorders (Franck et al., 2007a; Pedreschi et al., 2009), and in skin of apples treated with 5% CO_2 with and without 1-methylcyclopropene (Deewatthanawong and Watkins, 2010). GABA accumulated in CO_2 treated strawberries, but the maximum concentrations obtained were affected by cultivar (Deewatthanawong et al., 2010). Other stresses, such as salinity can increase GABA accumulation in tomato fruit (Zushi and Matsuzoe, 2007a; Saito et al., 2008).
Our interest in understanding the possible role of GABA in stress responses of fruit to postharvest treatments. The objective of this study is to investigate effects of elevated CO_2 on GABA metabolism in tomatoes; CO_2 concentrations higher than 3-5% can

induce injury in most cultivars (Grierson and Kader, 1986). In this paper, we present GABA responses to a 10% CO_2 treatment in breaker and red ripening stages at both the enzymatic and transcriptional levels. Changes in GABA, GAD activity, GABA-T activity, and gene expression of GABA shunt enzymes were measured during storage and after transfer of fruit to air.

4.2 MATERIALS AND METHODS

4.2.1 Plant material and treatments

Hydroponic cluster tomatoes (*Solanum lycopersicum* cv. Tricia) were obtained from a commercial greenhouse in Youngstown, NY. Fruit were harvested and immediately transported to the laboratory at Cornell University in Ithaca. Upon arrival, fruit were cooled at 13 ^{o}C for 6 h and then sorted according to developmental stage, and for uniformity in size. Fruit at different maturity stages (green, breaker, turning, pink and light red) were used for GABA analysis. Light red fruit are referred to as red fruit for all subsequent data presentation and discussion. To study the effects of CO_2, breaker fruit and red fruit (groups of six) were placed in a 19 L air tight plastic containers connected to a continuous flow-through gas system (Fawbush et al., 2008) with humidified air or air containing 10% CO_2 gas mixture. Atmospheres were monitored daily by gas chromatography (Fisher Gas Partitioner model 1200, Fisher Scientific, Springfield, NJ). Fruit were stored for 12 d at 13 ^{o}C, and transferred to air at 13 ^{o}C for a further 3 d. Six individual fruit were sampled from air or CO_2 treatments at 3 d intervals. Samples were withdrawn in the cold room immediately after containers were opened and each fruit was cut half vertically. The first half was immediately cut to smaller pieces. Five grams of pericarp was taken for enzyme analyses and the rest was frozen in liquid nitrogen for further analyses. The second half of the fruit was used for color assessment.

4.2.2 Fruit quality assessments

Tomato fruit color, as Chroma and Hue angles, was measured at three sites with a Minolta Chromameter CR-300 (Osaka, Japan). CO_2 injury was determined based on external damage (water-soaking, pitting, and decay) on each fruit using a subjective four-grade scoring system: 0 = no damage, 1 = slight damage, 2 = moderate damage, 3 = severe damage, and 4 = very severe damage.

4.2.3 GABA determination

GABA concentration in fruit pericarp was estimated by the method of Zhang and Bown (1997). Frozen pericarp was ground in liquid nitrogen to a fine powder, and 0.4 g of the ground tissue was extracted in 0.4 mL methanol for 10 min at room temperature. The mixture was vacuum dried with a Vacufuge Concentrator 5301 (Eppendorf, Hamburg, Germany). The dried sample was dissolved in 1 mL 70 mM lanthanum chloride followed by 15 min of shaking, and centrifugation at 13,000 x *g* for 5 min. The resulting supernatant was transferred to a new tube, and 0.8 mL supernatant was mixed with 0.16 mL 1 M potassium hydroxide. The mixture was shaken for 5 min, and centrifuged at 13,000 x *g* for 5 min. The supernatant was used for GABA determination.

GABA content was measured by UV spectrophotometry using GABase (Sigma, St. Louis, MO). The 1 mL assay mixture contained 0.6 mM $NADP^+$, 0.1 unit GABase, 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α-ketoglutarate and 550 µL sample. The reduction of NADP to NADPH was monitored at 340 nm after 10 min incubation at room temperature using a Genesys 5 spectrophotometer (Spectronic Instruments, Waltham, MA).

4.2.4 GAD and GABA-T activity determination

Enzyme extraction was carried out in a buffer containing 0.1 M Tris-Cl (pH 9.1), 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 5mM ethylenediaminetetraacetic

acid (EDTA), 0.5 mM pyridoxal phosphate (PLP) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Fresh tissue was added to the pre-cooled extraction buffer at a ratio of 1:3 (w/v) and homogenized at 4 ^{o}C. The homogenate was filtered through Miracloth, then centrifuged at 24,500 x *g* at 4 ^{o}C for 30 min. The supernatant was used for GAD and GABA-T determination.

GAD activity was determined using the method of Bartyzel et al. (2003b). The enzyme activity was assayed by incubating crude extract at 30 ^{o}C for 60 min in 400 µL assay mixture containing 0.1 M potassium phosphate buffer (pH 5.8), 40 µM PLP and 3 mM L-glutamate. The reaction was stopped by adding 0.1 mL of 0.5 M hydrochloric acid. The amount of GABA in samples was determined as described above. Enzyme activity was calculated as GABA produced, mmol/ kg of protein mass/s.

GABA-T activity was determined by the method of Ansari et al. (2005b). The enzyme activity was assayed in a 500 µL reaction mixture containing 50 mM Tris-Cl (pH 8.2), 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM PLP , 10% (v/v) glycerol, 16 mM GABA, 4 mM pyruvate and 200 µL crude extract. The mixture was incubated at 30 ^{o}C for 60 min, and stopped by adding 50 µL of 40 mM sulphosalicylic acid. The amount of alanine (Ala) formed during the incubation was determined using the enzymatic reaction of alanine dehydrogenase. The reaction was performed in a 1 mL mixture containing terminated sample, 50 mM sodium carbonate buffer (pH 10), 1.5 mM NAD^{+} and 0.02 units of L-alanine dehydrogenase. The reaction was incubated at 25 ^{o}C for 10 min, and then absorbance was read at 340 nm using a Genesys5 spectrophotometer. Enzyme activity was calculated as Ala produced, mmol/kg of protein mass/s.

Protein concentrations were determined using the Bradford assay (Sigma, St Louis, MO) according to the manufacturer's micro assay protocol. Protein

concentrations were determined by comparison to the bovine serum albumin (BSA) standard curve (1-10 mg L^{-1}).

4.2.5 Gene expression analysis by relative quantitative RT-PCR

Tomato pericarp was ground in liquid nitrogen and RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with some modifications. Phenol-chloroform extraction and additional chloroform extraction steps were added prior RNA precipitation. RNA was quantified using a spectrophotometer (ND-100, NanoDrop Technologies, Wilmington, DE). The A260/280 ratios of RNA samples were in the range of 1.8-2.0. The RNA was treated with RNase free DNase (Ambion, Austin, TX). RNA was re-quantified with the ND-100 Nanodrop spectrophotometer. The overall integrity of RNA was checked on 1.5% non-denaturing agarose gels as described by Tsai et al. (2004). cDNA was synthesized from 1 µg of total RNA using random hexamers and a Retroscript kit (Ambion, Austin, TX) as recommended by the manufacturer. The cDNA was then used as a template for PCR amplification.

PCR reactions were performed with an Eppendorf Mastercycler (Hamburg, Germany) in triplicates using gene specific primers (Table 4.1) and Quantum RNA 18S Internal Standards (Ambion, Austin, TX). One microliter from each 20 µL cDNA synthesis reaction was used in a PCR reaction containing 1.25 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.2 mM each dNTPs, 1.5 mM Mg Cl_2, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.4 µM gene specific primers, and a mixture of 18S primers and competimer. All PCR reactions were subjected to an initial denaturation at 94 ^oC for 2 min and 27-32 cycles of denaturation at 94 ^oC for 45 s, annealing at 50 or 54 ^oC for 45 s, and extension at 72 ^oC for 45 s, followed by the final extension at 72 ^oC for 15 min. The PCR cycle number and annealing temperature for each gene are shown in Table 1. Amplicons were separated and visualized on 1.5%

agarose gels containing ethidium bromide. The band intensity of the gene of interest and 18S internal standard in the same lane was quantified using Quantity One software (Version 4.6.2, BioRad, Hercules, CA). The signal intensity ratio between the target gene and 18S RNA was calculated to obtain the relative expression of the gene of interest.

Table 4.1 PCR primer sequences and PCR conditions used for the expression analysis of GABA shunt genes.

Accession No.	Primer	Primer sequence (5'-3')	Annealing temperature (^{o}C)	Number of cycles
AB359913	GAD1_F	GAAAAGAAAAGCAGAAGGCAAA	50	27
	GAD1_R	CATACTTGTGACCGCTGACATT		
AB359914	GAD2_F	CAGTTGGAGTTGGAACAGTTGG	50	27
	GAD2_R	CTCAGGGCTCATCACATAGTATCC		
AB359915	GAD3_F	CAAGACTTGCCTCAACAACTC	54	32
	GAD3_R	AGACAAGACGATCTGCTAGG		
AB359916.1	GABAT1_F	TCTGATGTAATTCATTCTCAAAGCA	50	30
	GABAT1_R	TCCCATAGATTCTAATCAACTCGTC		
AB359917	GABAT2_F	GCGGAGGGTTCTTATGTCTATG	50	30
	GABAT2_R	GTAGTGATGGAAGCCCAGAGAG		
AB359918	GABAT3_F	GGGGAAGCTGAGGCATATGTTA	50	30
	GABAT3_R	TCATGCCCCTTGTATCCTCT		
AB359921.1	SSADH_F	CTCGGTTGAAACTAGTCCTTCATTA	50	30
	SSADH_R	CTAAAGCTGTCCAAAACATGAAAGT		
AB359919.1	SSR1_F	GGAGGAAATAGGGTTTCTAGGG	50	30
	SSR1_R	CTTCTGCAGGAGTTTCTCCAAC		
AB359920	SSR2_F	TCCAGTATCAGGGTCCAAGAA	54	32
	SSR2_R	CCGCTAAACCCAGAGCTAGAC		

4.2.6 Statistical analysis

The MINITAB software release 15 (Minitab Inc., State College, PA) was used for analysis of the data. One-way ANOVA was performed on the CO_2 injury data, and other data were analyzed using the General Linear Model (GLM).

4. 3 RESULTS

4.3.1 GABA concentration in fruit of different maturities at harvest

GABA concentrations decreased as the fruit ripened on the vine, concentrations being 1.8, 1.5, 1.0, 0.8 and 0.5 mmol kg^{-1} in green, breaker, turning, pink and red fruit, respectively. Breaker and red stages were chosen for subsequent experiments on the effects of CO_2 on fruit metabolism.

4.3.2 Fruit quality

Hue angle values were higher in breaker than red fruit, while chroma values in red fruit were higher than that in breaker fruit (Figure 4.1). The hue angle of both maturity stages decreased during storage, and to a greater extent in air treated than CO_2 treated fruit reflecting faster fruit ripening in air storage. In breaker fruit, chroma values increased during storage, and to a greater extent in air treated than CO_2 treated fruit. In red fruit, chroma values remained unchanged in both treatments. After fruit removal to air for 3 d, the CO_2 injury rating for red fruit was 3.5, while that of breaker fruit was 0.33. No injury symptoms were detected in air treated fruit (data not shown).

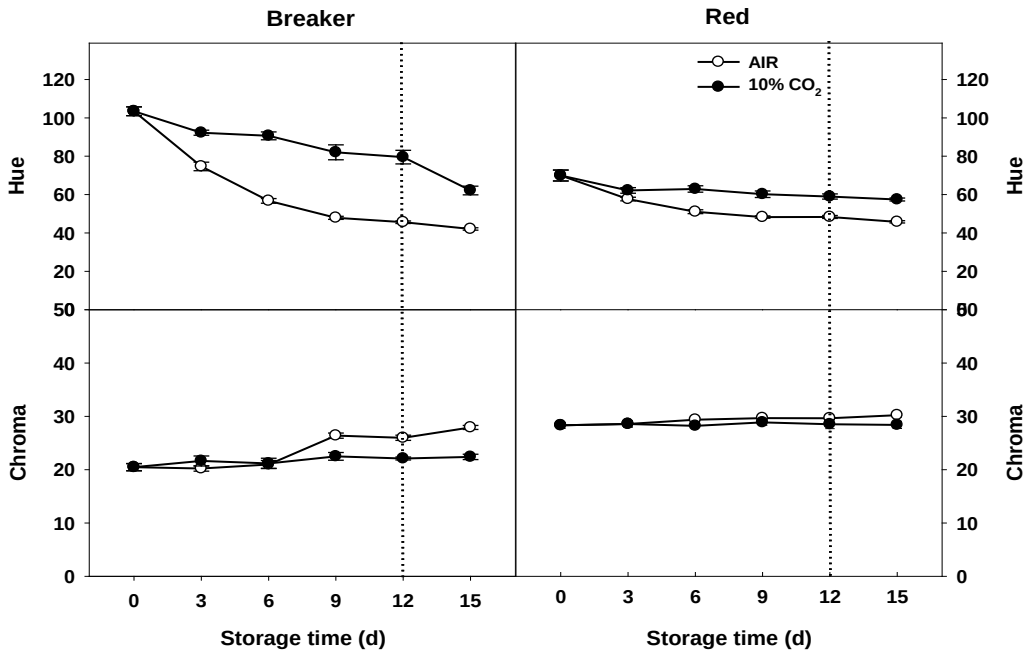
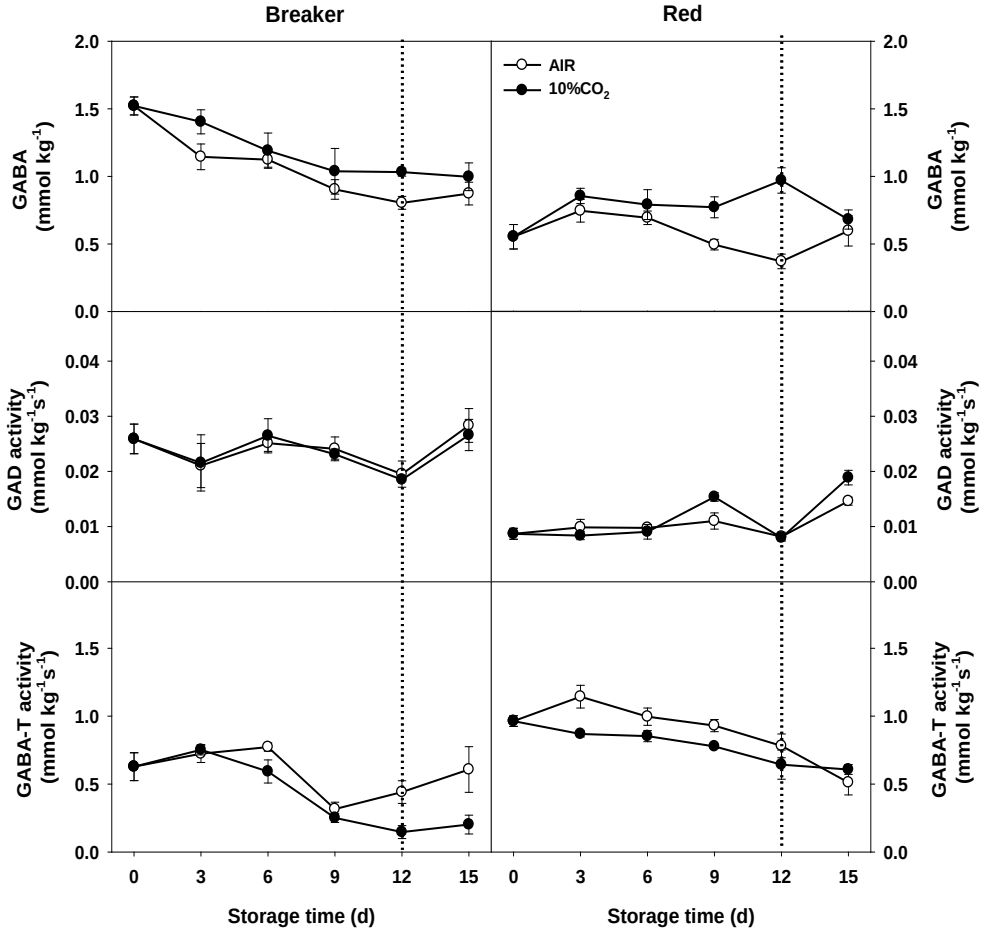


Figure 4.1 Hue angles and chroma values of breaker and red tomatoes stored in air or 10% CO_2 at 13 ^{o}C for 12 d followed by 3 d in air. The open and closed circles represent air storage and 10% CO_2 storage, respectively. Vertical dotted lines represent the time point when fruit were transferred to air. Standard errors are smaller than the symbols. For chroma values, maturity (m) and day (d) were significant at $P < 0.001$, while CA (c) was significant at $P = 0.001$. Interactions of m x d and d x c were significant at $P < 0.001$, while m x d x c was significant at $P = 0.014$. For hue angles, m, d, c, and their two-way and three-way interactions were significant at $P < 0.001$.

Figure 4.2 GABA concentrations, GAD activity and GABA-T activity, expressed on a protein mass basis, of breaker and red tomatoes stored in air or 10% CO_2 at 13 ^{o}C for 12 d followed by 3 d in air. The open and closed circles represent air storage and 10% CO_2 storage, respectively. Vertical dotted lines represent the time point when fruit were transferred to air. Standard errors are shown by vertical bars when they exceed the size of the symbol. For GABA, maturity (m), day (d), CA (c), and m x d were significant at $P <$ 0.001, while d x c was significant at $P = 0.015$. For GAD activity, s was significant at $P < 0.001$, while d was significant at $P = 0.012$. No other significant differences were detected. For GABA-TP activity, m, d, c, were significant at $P < 0.001$, while m x d was significant at $P = 0.005$. No other interactions were significant.


Breaker
Red
AIR
10%CO2
GABA (mmol kg-1)
GAD activity (mmol kg-1s-1)
GABA-T activity (mmol kg-1s-1)
Storage time (d)
Storage time (d)

4.3.3 GABA concentration

GABA concentrations decreased in both air and CO_2 treated breaker fruit during the storage period, but concentrations in CO_2 treated fruit averaged 1.2-fold higher than air treated fruit (Figure 4.2). By day 12, GABA concentrations in air and CO_2 treated fruit had decreased by 1.9-fold and 1.5-fold, respectively.

In red fruit, GABA concentrations increased during the first 3 d of storage in both air and CO_2 treated fruit, but then decreased in air treated fruit. GABA concentrations in air treated fruit were 1.5-fold lower by day 12. In contrast, GABA concentrations in CO_2 treated fruit remained relatively constant until day 9, and then increased by day 12. The concentration in CO_2 treated fruit was 1.8-fold higher than at harvest by day 12.

After removal of red fruit to air at 13 ^{o}C, GABA concentrations decreased in CO_2 treated fruit, but increased in air treated fruit. After the post-storage period, no significant differences were detected for either treatment in fruit of both maturity stages.

4.3.4 GABA shunt enzyme activities

At harvest, GAD activity in breaker fruit was 3-fold higher than in red fruit at harvest (Figure 4.2). GAD activity in breaker fruit fluctuated during the first 6 d of storage but decreased by 1.4-fold by day 12. Activity was not affected by treatment. In red fruit, GAD activity remained stable during storage except for a slight increase in CO_2 treated fruit on day 9. After transfer to air, GAD activity increased in all treatments in both stages.

At harvest, GABA-T activity in red fruit was 1.5-fold higher than in breaker fruit (Figure 4.2). GABA-T activity decreased during storage in all treatments, but activity was generally lower in CO_2 treated fruit than in air treated fruit. After transfer

to air, there were no significant changes in GABA-T activity in CO_2 treated fruit. Activity increased air treated breaker fruit but decreased in air treated red fruit.

4.3.5 Gene expression of GABA shunt enzymes

4.3.5.1 GAD

At harvest, the relative expression of *GAD1* and *GAD2* was 3.6-fold and 3.5-fold higher in breaker than in red fruit, respectively, whereas there was little effect of maturity stage on expression of *GAD3* (Figure 4.3).

GAD1 expression levels increased during storage of fruit of both maturity stages, but were not affected consistently by CO_2 treatment. There were no significant changes in relative gene expression of *GAD1* after transfer to air.

The relative expression of *GAD2* in breaker fruit, decreased slightly in air treated fruit but increased markedly in CO_2 treated fruit. In red fruit, *GAD2* expression also increased more in CO_2 treated fruit than air treated fruit, though to a relatively smaller extent than in breaker fruit. *GAD2* expression decreased after transfer from CO_2 to air, especially in breaker fruit.

The relative expression of *GAD3* increased in breaker fruit, but to a greater extent in CO_2 treated than air treated fruit where a decrease occurred after day 6. In red fruit, *GAD3* relative expression in CO_2 treated fruit remained unchanged during storage but decreased by 1.6-fold in air treated fruit.

Figure 4.3 Relative expression of *GAD1*, *GAD2*, and *GAD3* genes in air or 10% CO_2 of breaker or red tomatoes at 13 ^{o}C for 12 d followed by 3 d in air. The open and closed circles represent air storage and 10% CO_2 storage, respectively. Vertical dotted lines represent the time point when fruit were transferred to air. Standard errors are shown by vertical bars when they exceed the size of the symbol. For *GAD1*, maturity (m), day (d) were significant at P <0.001. No other differences were significant. For *GAD2*, d and CA (c) were significant at P <0.001, while m, m x c, and d x c were significant at P = 0.006, 0.007, and 0.002, respectively. For *GAD3*, m, c, and d x c were significant at P <0.001, while d was significant at P = 0.033. Interactions of m x d, m x c, and m x d x c were significant at P = 0.001, 0.002, and 0.024, respectively.

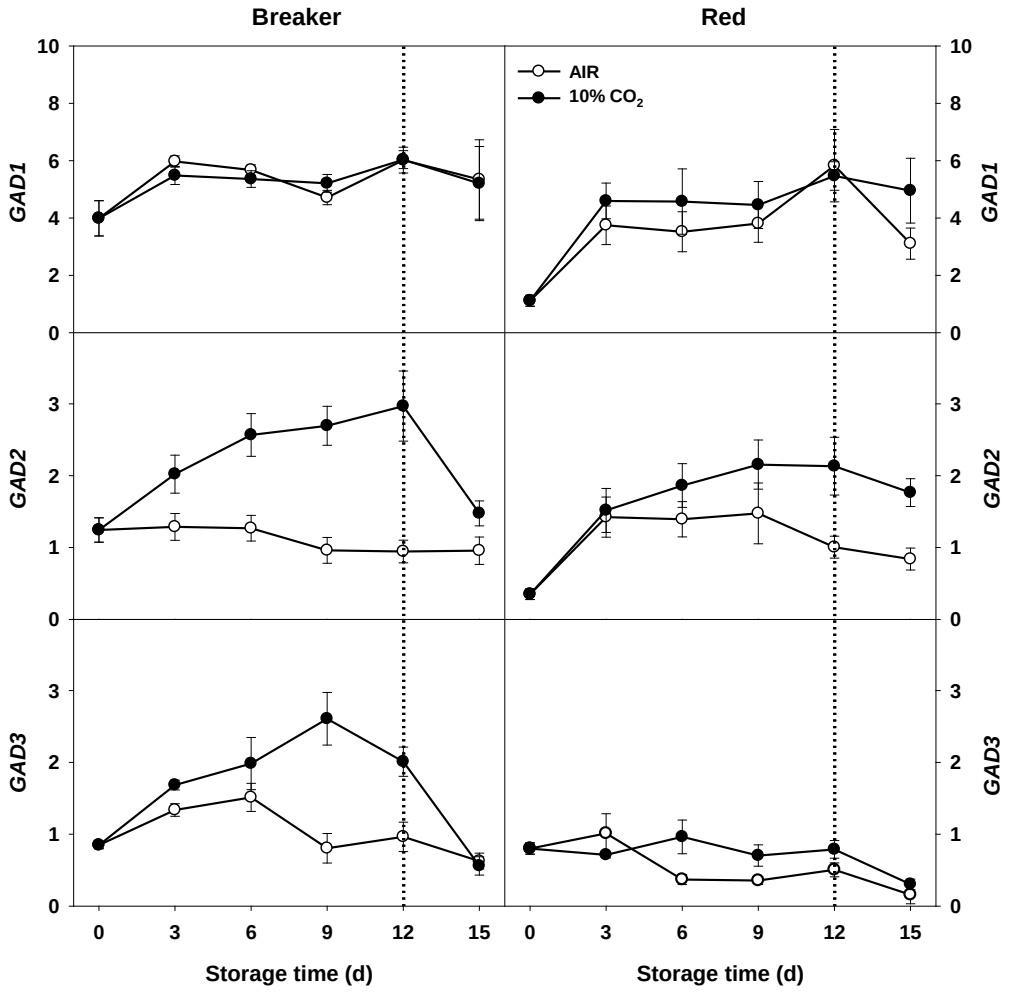
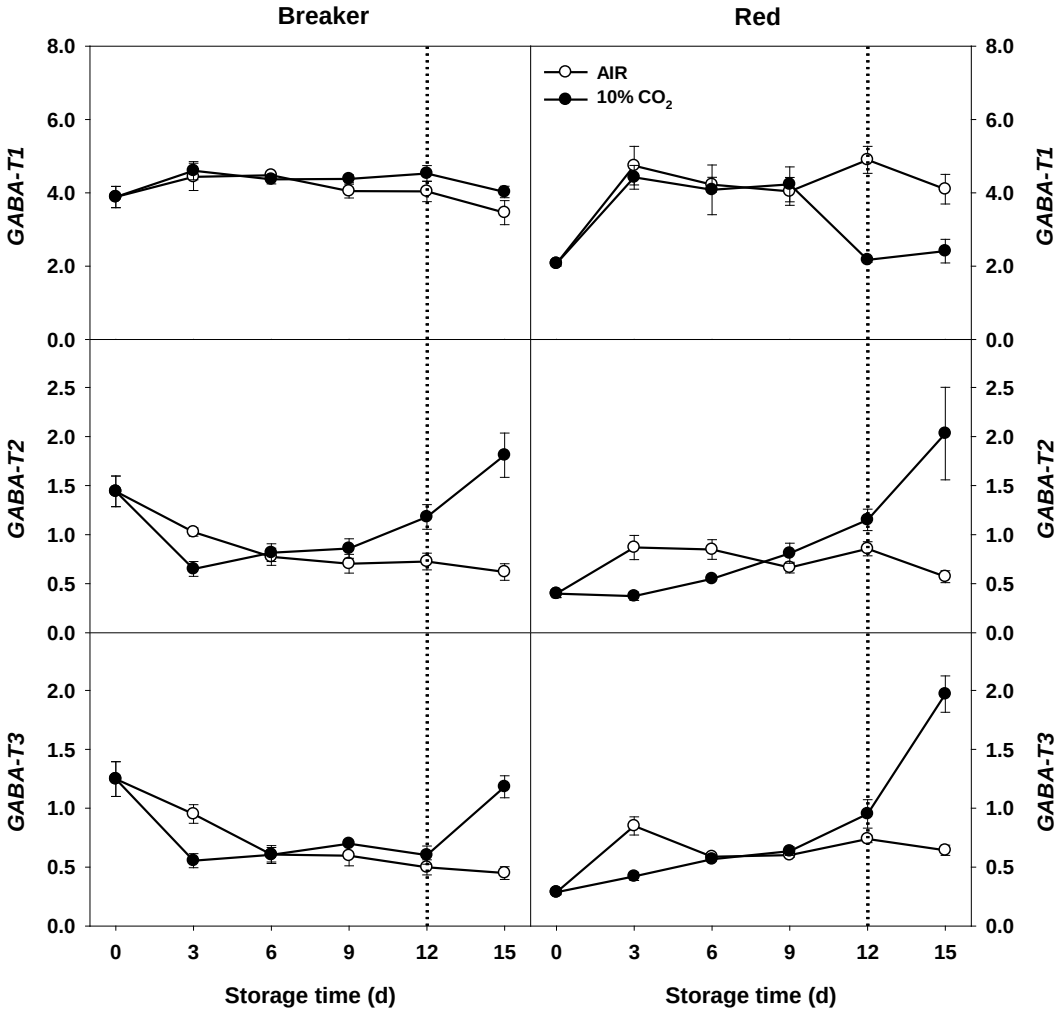

Breaker
Red
AIR
10% CO2
GAD1
GAD2
GAD3
Storage time (d)
Storage time (d)

Figure 4.4 Relative expression of *GABAT1*, *GABAT2*, and *GABAT3* genes in air or 10% CO_2 of breaker or red tomatoes at 13 ^{o}C for 12 d followed by 3 d in air. The open and closed circles represent air storage and 10% CO_2 storage, respectively. Vertical dotted lines represent the time point when fruit were transferred to air. Standard errors are shown by vertical bars when they exceed the size of the symbol. For *GABAT1*, differences in maturity (m) and day (d) were significant at $P < 0.001$, while no significant differences were detected in CA (c). Interactions of m x d, m x c, d x c, m x d x c were significant at $P = 0.001$, 0.009, 0.035, and 0.003, respectively. For *GABAT2*, m, m x d, and d x c were significant at $P < 0.001$, while d was significant at $P = 0.001$. No other differences were significant. For *GABAT3*, m, m x d, and d x c were significant at $P < 0.001$, while d was significant at $P = 0.018$. No other significant differences were found.


Breaker
Red
AIR
10% CO2
GABA-T1
GABA-T2
GABA-T3
Storage time (d)
Storage time (d)

4.3.5.2 GABA-T

The relative expressions of *GABA-T1*, *GABA-T2*, and *GABA-T3* were higher in breaker fruit than in red fruit at harvest (Figure 4.4). *GABA-T1* expression in breaker fruit remained unchanged over time and was not affected by treatment. In red fruit, *GABA-T1* expression increased initially and remained relatively stable until day 12 when a sharp decrease occurred in CO_2 treated fruit. There was little change in expression of *GABA-T1* after transfer to air.

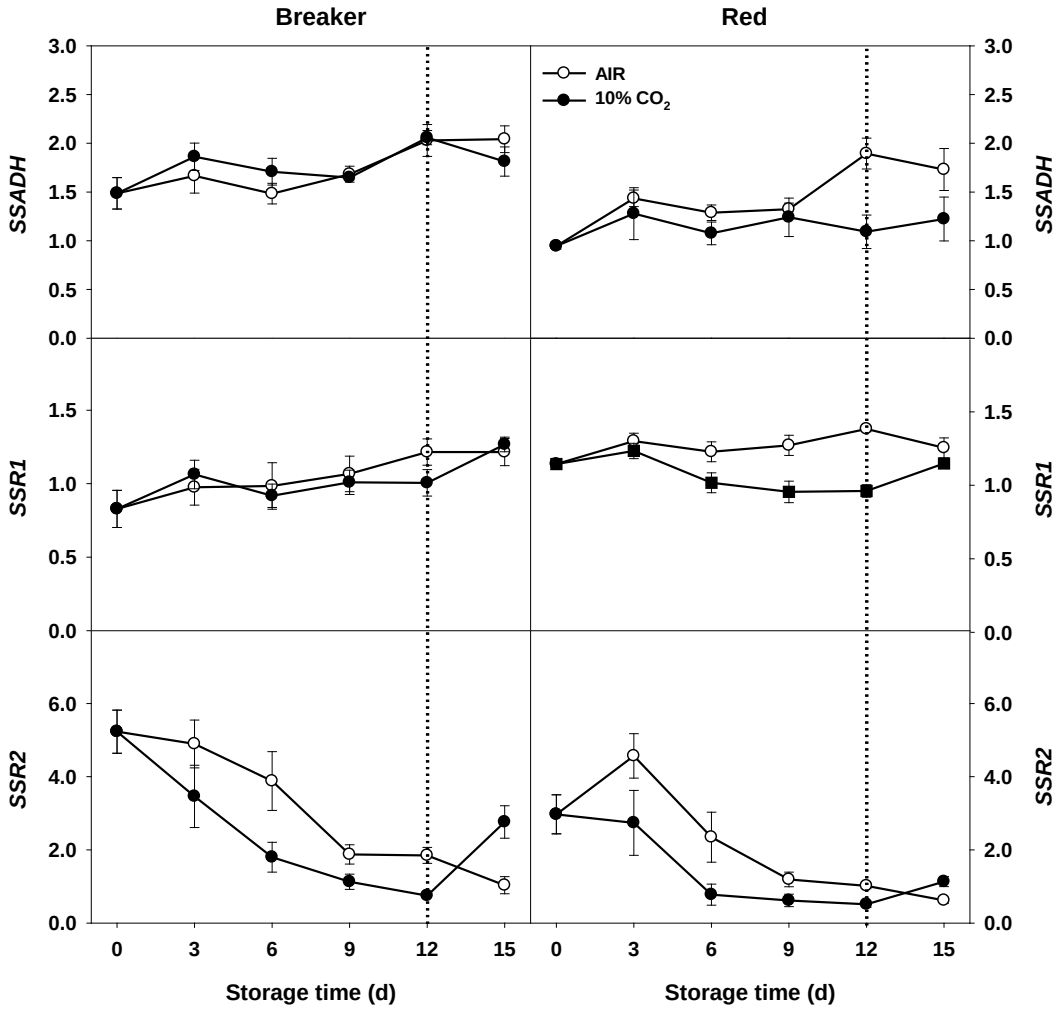
GABA-T2 expression decreased in both air and CO_2 treated breaker fruit during the first 3 d. Expression in air treated fruit continued to decline, but expression in CO_2 treated fruit increased with further storage. In red fruit, the relative expression of *GABA-T2* in CO_2 treated fruit increased during storage, while in air treated fruit, an increase occurred during the first 3 d of storage and then expression remained relatively stable. *GABA-T2* expression increased rapidly in CO_2 treated fruit after transfer to air.

The relative expression of *GABA-T3* decreased in breaker fruit from both treatments, but generally increased in red fruit. There was little effect of treatment on *GABA-T3* expression. However, *GABA-T3* expression increased sharply in CO_2 treated fruit of both maturity stages after transfer to air.

4.3.5.3 SSADH

Relative *SSADH* expression levels were higher in breaker fruit than in red fruit at harvest (Figure 4.5). Expression in breaker fruit increased during storage, but was not affected by treatment. In red fruit, expression was not affected by treatment during the first 9 d but increased in air treated fruit on day 12. No significant changes were observed in relative gene expression of *SSADH* after transfer to air.

Figure 4.5 Relative expression of *SSADH*, *SSR1*, and *SSR2* genes in air or 10% CO_2 of breaker or red tomatoes at 13 ^{o}C for 12 d followed by 3 d in air. The open and closed circles represent air storage and 10% CO_2 storage, respectively. Vertical dotted lines represent the time point when fruit were transferred to air. Standard errors are shown by vertical bars when they exceed the size of the symbol. For *SSADH*, differences in maturity (m) and day (d) were significant at $P < 0.001$, while no significant differences were detected in CA (c). Interaction of m x c was significant at $P = 0.009$. For *SSR1*, m was significant at $P < 0.001$, while c was significant at $P = 0.003$. No other significant differences were detected. For *SSR2*, m, d, and c were significant at $P < 0.001$. Interactions of main factors were not significant.


Breaker
Red
AIR
10% CO2
SSADH
SSR1
SSR2
Storage time (d)
Storage time (d)

4.3.5.4 SSR

The relative expression of *SSR1* gene was slightly higher in red fruit than breaker fruit at harvest (Figure 4.5). Breaker fruit from both treatments showed a slight increase in *SSR1* gene expression during storage. In red fruit, gene expression of *SSR1* increased 1.2-fold in air, but 1.2-fold lower in those treated with CO_2. Increased gene expression occurred in CO_2 treated breaker and red fruit after transfer to air.

SSR2 expression was greater in breaker than red fruit at harvest, but expression generally declined over storage time and was lower in CO_2 treated fruit of both maturity stages. A pronounced increase in relative expression of *SSR2* occurred in CO_2 treated breaker fruit after transfer to air.

4.4 DISCUSSION

GABA is a major amino acid during tomato fruit development, reaching maximum concentrations at the mature green/breaker maturity stages before declining as fruit ripen (Rolin et al., 2000; Kisaka et al., 2006b; Mounet et al., 2007; Akihiro et al., 2008b; Saito et al., 2008) GABA may be involved in maintenance of pH in the cytoplasm during organic acid accumulation during fruit development (Rolin et al., 2000) or provide transient nitrogen storage during the early stage of fruit development (Sata Narayan and Nair, 1990).

In this study, we have compared the effects of postharvest CO_2 storage on fruit at breaker and red maturity stages. The ripening of breaker stage fruit was inhibited to a greater extent than the red fruit as judged by hue and chroma values (Figure1), but the red fruit were more sensitive to development of CO_2 injury than the breaker fruit. GABA concentrations in breaker fruit decreased during storage in both treatments, but

to a lesser extent in CO_2 treated than air treated fruit (Figure 2). In contrast, GABA initially accumulated in red fruit in both air and CO_2, and then declined in air, while increasing slightly during the CO_2 treatment. The accumulation of GABA in response to CO_2 treatment appears modest in view of a sharp increase in expression of GAD genes in mature green tomatoes treated with 20% CO_2 for 3 d (Rothan et al., 1997). However, GABA concentrations were not measured in that study. Makino et al. (2008) found that GABA accumulated in tomatoes exposed to modified atmosphere packaging (MAP) that resulted when exposed to 11% O_2 and 9% CO_2, but only at 30 ^{o}C; although similar patterns of GABA accumulation occurred at 15 ^{o}C as at 30 ^{o}C, the changes were not statistically significant. It is unknown if effects of CO_2 on GABA concentrations in tomato are affected by cultivar as shown for strawberry (Deewatthanawong et al., 2010). Interestingly, tomato fruit contain one of the highest GABA concentrations of all vegetable crops (Saito et al., 2008). Little data on GABA concentrations in non-vegetable fruit are available, but reported concentrations at harvest are much lower compared with tomato, and increase to a much greater extent when exposed to stress. In cherimoya fruit, GABA concentrations were about 0.05 mmol kg^{-1} at the start of treatment and accumulated to reach 0.45 mmol kg^{-1} (9-fold increase) after 3 d treatment with 20% CO_2 (Merodio et al., 1998a). GABA concentrations increased from 0.03 to 0.3 mmol kg^{-1} (10-fold increase) in the skin of 1-methylcyclopropene treated apples exposed to 5% CO_2 (Deewatthanawong and Watkins, 2010), while in strawberries, GABA concentrations in the most responsive cultivar, 'Northeaster', reached 1.7 mmol kg^{-1} from 0.2 mmol kg^{-1} at harvest (8.5-fold increase) after 12 d in 20% CO_2 (Deewatthanawong et al., 2010). In contrast the increase in MAP treated tomatoes was from 3.3 to 5.2 mmol kg^{-1} (1.6-fold) in the study of Makino et al. (2008) and from 0.5 to 1.0 mmol kg^{-1} (1.8-fold) for red stage tomatoes in our study (Figure 2).

GAD activity was higher in breaker than red fruit at harvest and it decreased during storage (Figure 2). This is consistent with a previous study in which GAD activity decreased after the breaker stage, and in which GABA rich varieties had highest GAD activity (Akihiro et al., 2008b). In our study, CO_2 did not affect GAD activity, but was associated with lower GABA-T activity (Figure 2). This result suggests that higher GABA concentrations in these fruit may be due to a lower rate of GABA degradation, rather than higher GABA production associated with increased GAD activity.

GAD activity was assayed at a pH of 5.8, within the common range of pH used in assays for this enzyme in tomato, potato and other tissues (Satya Narayan and Nair, 1990b; Rolin et al., 2000; Bartyzel et al., 2003b; Li et al., 2010), even though GAD is a cytoplasmic enzyme. In cherry tomato, GAD activity at pH 7.0 was only 15% of the maximal activity at pH 6.0 (Rolin et al, 2000), although GAD activity was measured at pH 7 by Akihiro et al. (2008). GAD is regulated by pH and calcium/calmodulin activation (Gallego et al., 1995; Baum et al., 1996a; Snedde et al., 1996; Bouche et al., 2004a; Oh et al., 2005b), and the two levels of regulation are independent (Gut et al., 2009). When pH drops, GAD activity is enhanced, while at neutral pH, GAD is mediated by calmodulin binding (Snedden et al., 1995b; Gut et al., 2009). High CO_2 stress conditions may cause cellular acidification in harvested plant tissues (Siriphanich and Kader, 1986; Lange and Kader, 1997) and increased cellular calcium levels (Wheeler et al., 1997; Sanders et al., 2002), which in turn may activate GABA synthesis by GAD (Kinnersley and Turano, 2000). Further study of GAD activity at different pH levels in CO_2 treated tomato fruit is warranted.

Analysis of gene expression, however, indicated that the effects of CO_2 on GAD may not be reflected by measurement of total enzyme activity. The expression of the *GAD1* gene was not affected by CO_2 treatment, but that of *GAD2* and *GAD3*

was greater in CO_2 treated than air treated fruit (Figure 3). Interestingly, Kisaka et al. (2006) demonstrated that GAD antisense tomatoes had lower expression of GAD mRNA, but not all plants had lower GABA concentrations. Also, the differences between GABA concentrations in transformed and wild type plants were not significant. A possible explanation is that there are multiple *GAD* isoforms in each species and each isoform tends to have specific function and regulation.

GABA-T activity was lower in CO_2 treated fruit, especially at the red maturity stage (Figure 2), suggesting that higher GABA concentrations in these fruit may be due to a lower rate of GABA degradation. Only pyruvate-dependent GABA-T (GABA-TP) activity was measured in the current study. Akihiro et al. (2008) found that α-ketoglutarate-dependent GABA-T (GABA-TK) activity was much greater than GABA-TP activity, and suggested that GABA-TK played an important role in GABA catabolism in tomatoes. However, while Clark et al. (2009) identified cytosol- and plastid-localized GABA-T isoforms, they were unable to reproduce the results showing activity of GABA-TK as found by Akihiro et al. (2008). Clark et al. (2009) concluded that pyruvate/glyoxylate dependent GABA-T activity probably accounts for GABA degradation.

Consistent effects of high CO_2 on expression of the *GABA-Ts* and *SSADH* expression by high CO_2 stress were not observed (Figs. 4 and 5). However, CO_2 treatments lowered *SSR2* expression in breaker fruit, and lowered *SSR1* and *SSR2* expression in red fruit. If effects on expression are directly related to enzyme activity, lower transcript levels in CO_2 treated red fruit could result in accumulation of SSA in the fruit, which is a toxic metabolite that would damage cells under stress conditions. After removal of fruit from the storage, expression of *GAD2* and *GAD3* decreased, but *GABA-T3* and *SSR1* and *SSR2* increased in CO_2 treated fruit of both stages. It is

possible that when the stress is relieved, GABA may be metabolized by GABA-T and then SSA may be reduced in the cytosol via SSR reactions.

In conclusion, GABA accumulated more in red fruit than in breaker fruit in response to CO_2 treatment, but whether or not this accumulation is associated with greater sensitivity of fruit to high CO_2 stress is uncertain. GABA accumulation in red fruit was probably due to an increase in GABA degradation including lower GABA-T activity and expressions of some degradation enzymes. However, more information is needed on other GABA shunt intermediates, related enzyme activities, and isozyme studies.

REFERENCES

Abe, Y., Umemura, S., Sugimoto, K., Hirawa, N., Kato, Y., Yokoyama, N., Yokoyama, T., Iwai, J., Ishii, M., 1995. Effect of green tea rich in γ-aminobutyric acid on blood pressure of Dahl salt-sensitive rats. Am. J. Hypertens. 8, 74-79.

Akihiro, T., Koike, S., Tani, R., Tominaga, T., Watanabe, S., Iijima, Y., Aoki, K., Shibata, D., Ashihara, H., Matsukura, C., Akama, K., Fujimura, T., Ezura, H., 2008. Biochemical mechanism on GABA accumulation during fruit development in tomato. Plant Cell Physiol. 49, 1378-1389.

Allan, W.L., Peiris, C., Bown, A.W., Shelp, B.J., 2003. Gamma-hydroxybutyrate accumulates in green tea and soybean sprouts in response to oxygen deficiency. Can. J. Plant Sci. 83, 951-953.

Allan, W.L., Simpson, J.P., Clark, S.M., Shelp, B.J., 2008. γ-Hydroxybutyrate accumulation in *Arabidopsis* and tobacco plants is a general response to abiotic stress: putative regulation by redox balance and glyoxylate reductase isoforms. J. Exp. Bot. 59, 2555–2564.

Ansari, M.I., Lee, R.H., Chen, S.C.G., 2005. A novel senescence-associated gene encoding γ-aminobutyric acid (GABA):pyruvate transaminase is upregulated during rice leaf senescence. Physiol. Plant. 123, 1-8.

Antonaccio, M.J., Taylor, D.G., 1977. Involvement of central GABA receptors in the regulation of blood pressure and heart rate of anesthetized cats. Eur. J. Clin. Nutr. 46, 283-287.

Bartyzel, I., Pelczar, K., Paszkowski, A., 2003. Functioning of the γ-aminobutyrate pathway in wheat seedlings affected by osmotic stress. Biol. Plant. 47, 221-225.

Baum, G., Lev-Yadun, S., Fridmann, Y., Arazi, T., Katsnelson, H., Zik, M., Fromm, H., 1996. Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. EMBO J. 15, 2988-2996.

Bouche, N., Lacombe, B., Fromm, H., 2003. GABA signaling: a conserved and ubiquitous mechanism. Trends Cell Biol. 13, 607-610.

Bouche, N., Fait, A., Zik, M., Fromm, H., 2004. The root-specific glutamate decarboxylase (GAD1) is essential for sustaining GABA levels in *Arabidopsis*. Plant Mol. Biol. 55, 315-325.

Bouche, N., Fromm, H., 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9, 110-115.

Bowery, N.G., Smart, T.G., 2006. GABA and glycine as neurotransmitters: a brief history. Br. J. Pharmacol. 147, S109-S119.

Breitkreuz, K.E., Allan, W.L., Cauwenberghe, O.R.V., Jakobs, C., Talibi, D., B., A., Shelp, B.J., 2003. A novel γ-hydroxybutyrate dehydrogenase. Identification and expression of an *Arabidopsis* cDNA and potential role under oxygen deficiency. J. Biol. Chem. 278, 41552-41556.

Clark, S.M., Leo, R.D., Van Cauwenberghe, O.R., Mullen, R.T., Shelp, B.J., 2009. Subcellular localization and expression of multiple tomato γ-aminobutyrate transaminases that utilize both pyruvate and glyoxylate. J. Exp. Bot. 60, 3255-3267.

Deewatthanawong, R., Nock, J.F., Watkins, C.B., 2010. γ-Aminobutyric acid (GABA) accumulation in four strawberry cultivars in response to elevated CO_2 storage. Postharvest Biol. Technol. 57, 92-96.

Deewatthanawong, R., Watkins, C.B., 2010. Accumulation of γ-aminobutyric acid in apple, strawberry and tomato fruit in response to postharvest treatments. Acta Hort. in press.

Fait, A., Yellin, A., Fromm, H., 2005. GABA shunt deficiencies and accumulation of reactive oxygen intermediates: insight from *Arabidopsis* mutants. FEBS Lett. 579, 415-420.

Fait, A., Fromm, H., Walter, D., Galili, G., Fernie, A.R., 2007. Highway or byway: the metabolic role of the GABA shunt in plants. Trend Plant Sci. 13, 14-19.

Fawbush, F., Nock, J.F., Watkins, C.B., 2008. External carbon dioxide injury and 1-methylcyclopropene (1-MCP) in the 'Empire' apple. Postharvest Biol. Technol. 48, 92-98.

Franck, C., Lammertyn, J., Tri Ho, Q., Verboven, P., Verlinden, B., Nicolaï, B.M., 2007. Browning disorders in pear fruit. Postharvest Biol. Technol. 43, 1-13.

Gallego, P.P., Whotton, L., Picton, S., Grierson, D., Gray, J.E., 1995. A role for glutamate decarboxylase during tomato ripening: the characterisation of a cDNA encoding a putative glutamate decarboxylase with a calmodulin-binding site. Plant Mol. Biol. 27, 1143-1151.

Grierson, D., Kader, A.A., 1986. Fruit ripening and quality. In: Atherton, J.G., Rudich, J. (Eds.), The tomato crop: A scientific basic for improvement. Chapman and Hall, London. pp. 661.

Gut, H., Dominici, P., Pilati, S., Astegno, A., Petoukhov, M.V., Svergun, D.I., Grütter, M.G., Capitani, G., 2009. A common structural basis for pH- and calmodulin-mediated regulation in plant glutamate decarboxylase. J. Mol. Biol. 392, 334-351.

Kinnersley, A.M., Turano, F.J., 2000. Gamma aminobutyric acid (GABA) and plant responses to stress. Crit. Rev. Plant Sci. 19, 479-509.

Kisaka, H., Kida, T., Miwa, T., 2006. Antisense suppression of glutamate decarboxylase in tomato (*Lycopersicon esculentum* L.) results in accumulation of glutamate in transgenic tomato fruits. Plant Biotech. 23, 267-274.

Lancien, M., Roberts, M.R., 2006. Regulation of *Arabidopsis thaliana* 14-3-3 gene expression by γ-aminobutyric acid. Plant Cell Environ. 29, 1430-1430.

Lange, D.L., Kader, A.A., 1997. Elevated carbon dioxide exposure alters intracellular pH and energy charge in avocado fruit tissue. J. Am. Soc. Hort. Sci. 122, 253-257.

Li, Y., Bai, Q., Jin, X., Wen, H., Gu, Z., 2010. Effects of cultivar and culture conditions on γ -aminobutyric acid accumulation in germinated fava beans (*Vicia faba* L.). J Sci. Food Agric. 90, 52-57.

Makino, Y., Soga, N., Oshita, S., Kawagoe, Y., Tanaka, A., 2008. Stimulation of γ-aminobutyric acid production in vine-ripe tomato (*Lycopersicon esculentum* Mill.) fruits under modified atmospheres. J. Agri. Food Chem. 56, 7189-7193.

Matumoto, Y., Ohno, K., Hiraoka, Y., 1997. Studies on the utilization of functional food materials containing high levels of gamma-aminobutyric acid (Part 1). Ehime Kougi Kenkyu Houkoku 35, 97-100.

Merodio, C., Munoz, M.T., Cura, B.D., Buitrago, D., Escribano, M.I., 1998. Effect of high CO_2 level on the titres of γ-aminobutyric acid, total polyamines and some pathogenesis-related proteins in cherimoya fruit stored at low temperature. J. Exp. Bot. 49, 1339-1347.

Mounet, F., Lemaire-Chamley, M., Maucourt, M., Cabasson, C., Giraudel, J.L., Deborde, C., Lessire, R., Gallusci, P., Bertrand, A., Gaudillere, M., Rothan, C., Rolin, D., Moing, A., 2007. Quantitative metabolic profiles of tomato flesh and seeds during fruit development: complementary analysis with ANN and PCA. Metabolomics 3, 273-288.

Oh, S.H., Choi, W.G., Lee, I.T., Yun, S.J., 2005. Cloning and characterization of a rice cDNA encoding glutamate decarboxylase. J. Biochem. Mol. Biol. 38, 595-601.

Pedreschi, R., Franck, C., Lammerteyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123-130.

Rolin, D., Baldet, P., Just, D., Chevalier, C., Biran, M., Raymond, P., 2000. NMR study of low subcellular pH during the development of cherry tomato fruit. Aust. J. Plant Physiol. 27, 61-69.

Rothan, C., Duret, S., Chevalier, C., Raymond, P., 1997. Suppression of ripening-associated gene expression in tomato fruits subjected to a high CO_2 concentration. Plant Physiol. 114, 255.263.

Saito, T., Matsukura, C., Sugiyama, M., Watahiki, A., Ohshima, I., Iijima, Y., Konishi, C., Fujii, T., Inai, S., Fukuda, N., Nishimura, S., Ezura, H., 2008. Screening for γ-aminobutyric acid (GABA)-rich tomato varieties. J. Japan. Soc. Hort. Sci. 77, 242-250.

Sanders, D., Pelloux, J., Brownlee, C., Harper, J.F., 2002. Calcium at the crossroads of signaling. Plant Cell 14, S401-S417.

Satya Narayan, V., Nair, P.M., 1990. Metabolism, enzymology and possible role of 4-aminobutyric acid in higher plants. Phytochemistry 29, 367-375.

Shelp, B.J., Bown, A.W., McLean, M.D., 1999. Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446-452.

Shimada, M., Hasegawa, T., Nishimura, C., Kan, H., Kanno, T., Nakamura, T., Matsubayashi, T., 2009. Anti-hypertensive effect of γ-Aminobutyric Acid (GABA)-rich chlorella on high-normal blood pressure and borderline hypertension in placebo-controlled double blind study Clin. Exp. Hypertens. 31, 342 - 354

Siriphanich, J., Kader, A.A., 1986. Changes in cytoplasmic and vacuolar pH in harvested lettuce tissue as influenced by CO_2. J. Am. Soc. Hort. Sci. 111, 73-77.

Snedden, W.A., Koutsia, N., Baum, G., Fromm, H., 1995. Calcium/calmodulin activation of soybean glutamate decarboxylase. Plant Physiol. 108, 543-549.

Snedden, W.A., Koutsia, N., Baum, G., Fromm, H., 1996. Activation of a recombinant petunia glutamate decarboxylase by calcium/calmodulin or by a monoclonal antibody which recognizes the calmodulin binding domain. J Biol. Chem. 271, 4148-4153.

Tsai, C.J., Cseke, L.J., Harding, S.A., 2004. Isolation and purification of RNA. In: Csek, L.J., Kaufman, P.B., Podila, G.K., Tsai, C.J. (Eds.), Handbook of molecular and cellular methods in biology and medicine. CRC Press, Boca Raton, FL. pp. 25-44.

Wheeler, R.M., Mackowiak, C.L., Stutte, G.W., Yorio, N.C., Berry, W.L., 1997. Effect of elevated carbon dioxide on nutritional quality of tomato. Adv. Space Res. 20, 1975-1978.

Yamakoshi, J., Fukuda, S., Satoh, T., Tsuji, R., Saito, M., Obata, A., Matsuyama, A., Kikuchi, M., Kawasaki, T., 2007. Antihypertensive and natriuretic effects of less-sodium soy sauce containing γ-aminobutyric acid in spontaneously hypertensive rats. Biosci. Biotechnol. Biochem. 71, 165-173.

Yoshimura, M., Toyoshi, T., Sano, A., Izumi, T., Fuji, T., Konishi, C., Inai, S., Matsukura, C., Fukuda, N., Ezura, H., Obata, A., 2010. Antihypertensive effect of a γ-aminobutyric acid rich tomato cultivar 'DG03-9' in spontaneously hypertensive rats. J. Agric. Food Chem. 58, 615-619.

Zhang, G., Bown, A.W., 1997. The rapid determination of γ-aminobutyric acid. Phytochemistry 44, 1007-1009.

Zushi, K., Matsuzoe, N., 2007. Salt stress-enhanced γ-aminobutyric acid (GABA) in tomato fruit. Acta Hort. 761, 431-436.

CHAPTER 5

γ- AMINOBUTYRIC ACID (GABA) ACCUMULATION IN FOUR STRAWBERRY CULTIVARS IN RESPONSE TO ELEVATED CO_2 STORAGE

ABSTRACT

Accumulation of γ-aminobutyric acid (GABA) is associated with stress factors in plant systems. The objective of the current study was to compare GABA concentrations in four strawberry (*Fragaria x ananassa* Duch) cultivars with different tolerances to postharvest CO_2 treatment (20% in air) as indicated by accumulation of fermentation products. Color change of fruit of all cultivars was delayed by CO_2 treatment. Concentrations of ethanol, and ethyl acetate increased in CO_2 treated fruit of 'Jewel' but not in 'Allstar', 'Earliglow', and 'Northeaster'. Higher GABA concentrations were associated with elevated CO_2 treatment in all cultivars compared with air, but GABA accumulations were much lower in 'Allstar' and 'Earliglow' than in 'Jewel' and 'Northeaster'. At harvest, glutamate decarboxylase (GAD) activity was greater in fruit of 'Jewel' and 'Northeaster' than in 'Allstar' and 'Earliglow'. GAD activity decreased during storage, but it was not affected by CO_2. GABA transaminase (GABA-T) activity was lower in CO_2 than air treated 'Jewel' fruit, but it was not affected consistently by CO_2 in the other cultivars. The results indicate that high CO_2 treatments increased GABA concentrations in strawberry fruit, but the accumulation is not consistently associated with sensitivity of the fruit to CO_2 as indicated by accumulation of fermentation products.

5.1 INTRODUCTION

The strawberry fruit is relatively tolerant to elevated CO_2 in the storage atmosphere, and concentrations of 15-20% are used routinely by some industries to maintain flesh firmness and reduce decay (Mitcham, 2004). However, depending on cultivar, strawberries can accumulate the fermentation products, acetaldehyde, ethanol and ethyl acetate (EA), in response to elevated CO_2 (Fernandez-Trujillo et al., 1999; Watkins et al., 1999; Ponce-Valadez and Watkins, 2008). While commercially, accumulation of fermentation products is undesirable, such differences in fermentative response to CO2 among cultivars reflect metabolic differences in responses to stress imposed by postharvest treatment (Ponce-Valadez and Watkins, 2008).

Accumulation of γ-aminobutyric acid (GABA) is a metabolic response of plant systems to stress such as salinity, anoxia, hypoxia, drought, heat, and chilling (Streeter and Thompson, 1972; Wallace et al., 1984; Tsushida and Murai, 1987; Mayer et al., 1990; Aurisano et al., 1995; Kinnersley and Turano, 2000; Zushi and Matsuzoe, 2007). GABA is an intermediate involved in bypassing two steps of the tricarboxylic acid (TCA) cycle (Balazs et al., 1970), that is synthesized by glutamate decarboxylase (GAD). GABA is catabolized by GABA transaminase (GABA-T) to succinic semialdehyde, and subsequently to succinate by succinate dehydrogenase (SSADH) (Bown and Shelp, 1989). In tomatoes, GABA accumulation was affected by cultivar, and a salinity-tolerant cultivar had the smallest GABA accumulation (Saito et al., 2008). GABA and the shunt pathway is also involved in other physiological processes such as pH-stat, nitrogen storage, plant development, carbon metabolism, plant defense against insects, osmoregulation, and also functions in signal transduction (Breitkreuza et al., 1999; Shelp et al., 1999; Kinnersley and Turano, 2000; Bouche et al., 2003; MacGregor et al., 2003; McLean et al., 2003; Bouche and Fromm, 2004).

Information about postharvest GABA accumulation in fruit is limited. GABA was found to accumulate in 20% CO_2 stored cherimoya fruit (Merodio et al., 1998), in modified atmosphere (11% O_2 and 9% CO_2) stored tomatoes at 30 ^oC (Makino et al., 2008), and in pears stored in 1% O_2 and 10% CO_2 that developed flesh browning disorders (Franck et al., 2007; Pedreschi et al., 2009), but its role in stress responses during storage is not known.

The objective of this study was to determine whether the accumulation of GABA in strawberry is associated with tolerance of fruit to elevated CO_2 as indicated by accumulation of fermentation products in four cultivars. GABA, glutamate decarboxylase (GAD) activity and GABA transaminase (GABA-T) were determined to reveal possible relationship between GABA accumulation and sensitivity of fruit to high CO_2 stress.

5.2 MATERIALS AND METHODS

5.2.1 Plant material and treatments

Fruit of 'Allstar', 'Earliglow', 'Jewel', and 'Northeaster' strawberries (*Fragaria x ananassa* Duch) were obtained from a local farm in Locke, NY. Fruit were harvested at the orange stage of maturity and immediately transported to the laboratory at Cornell University in Ithaca. Berries were sorted to ensure uniformity in size, and to remove any damage fruit. After fruit (5 fruit replicates) were placed into 950-mL glass jars they were cooled at 2 ^oC for 3 h prior to atmosphere treatments. Atmospheres were applied by connecting the air tight glass jars to a continuous flow-through gas system with humidified air or air containing 20% CO_2 in air at a flow rate of 50 mL min^{-1} (Fernandez-Trujillo et al., 2007). Atmospheres were monitored twice daily using gas chromatography (Fisher Gas Partitioner model 1200, Fisher Scientific,

Springfield, NJ). Fruit were sampled at harvest and at 3 d intervals for 12 d. Each treatment was replicated five times at each sampling point.

5.2.2 Fruit color measurement

Fruit color was measured at 3 locations of each fruit using a Minolta Chromameter CR-300 (Osaka, Japan). Results were expressed as L* (brightness) value, chroma (color intensity) and hue angle (actual color) as described by Little (1975).

5.2.3 GABA determination

GABA concentration was determined using the method described by Zhang and Bown (1997). Strawberry fruit tissue was ground in liquid nitrogen to a fine powder. Ground tissue (0.4 g) was added to 0.4 mL methanol, mixed by votexing, and then left at room temperature for 10 min. The extract was vacuum dried with a Vacufuge Concentrator 5301 (Eppendorf, Hamburg, Germany). One milliliters of 70 mM lanthanum chloride was added to the dried sample, followed by 15 min shaking, and centrifugation at 13,000 *g* for 5 min. The supernatant (0.8 mL) was transferred to a new tube and mixed with 0.16 mL 1 M potassium hydroxide. The mixture was then shaken for 5 min, and centrifuged at 13,000 *g* for 5 min. The supernatant was used for GABA determination, using a GABase (Sigma, St. Louis, MO). The 1 mL reaction contained 0.6 mM $NADP^+$, 0.1 unit GABase, 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α-ketoglutarate and 550 μL sample. The absorbance at 340 nm was monitored before and after adding α-ketoglutarate for 10 min at room temperature using a Genesys 5 spectrophotometer (Spectronic Instruments, Waltham, MA).

5.2.4 GAD and GABA-T activity determination

Five grams of fresh tissue was added to 15 mL of extraction buffer containing 0.1 M Tris-Cl (pH 9.1), 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 5mM

ethylenediaminetetraacetic acid (EDTA), 0.5 mM pyridoxal phosphate (PLP) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was homogenized at 4 ^{o}C for 1 min. The homogenate was filtered through Miracloth, and then centrifuged at 24,500 *g* at 4 ^{o}C for 30 min. The supernatant was used for measurement of GAD and GABA-T activities.

GAD activity was determined according to the method of Bartyzel et al. (2003). The crude extract was incubated at 30 ^{o}C for 60 min in the 400 μL assay mixture containing 0.1 M potassium phosphate buffer (pH 5.8), 40 μM PLP and 3 mM L-glutamate. The reaction was terminated by adding 0.1 mL of 0.5 M hydrochloric acid. GABA concentrations in each sample were determined as described above.

GABA-T activity was determined using the method of Ansari et al. (2005). The enzyme activity was analyzed in a 500 μL reaction mixture containing 50 mM Tris-Cl (pH 8.2), 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM PLP , 10% (v/v) glycerol, 16 mM GABA, 4 mM pyruvate and 200 μL crude extract. The mixture was incubated at 30 ^{o}C for 60 min, and terminated by adding 50 μL of 40 mM sulphosalicylic acid. Alanine concentrations in each sample were determined using the enzymatic reaction of alanine dehydrogenase. The 1 mL reaction mixture contained the terminated sample, 50 mM sodium carbonate buffer (pH 10), 1.5 mM NAD^{+} and 0.02 units of L-alanine dehydrogenase. The reaction mixture was incubated at 25 ^{o}C for 10 min, and absorbance was read at 340 nm using a Genesys5 spectrophotometer.

Protein concentrations were measured using the Bradford reagent (Sigma, Saint Louis, MO) according to the manufacturer's microassay protocol. Protein concentrations were calculated using the standard curve of bovine serum albumin (1-10 μg/ml).

5.2.5 Volatile analysis

Ten grams of frozen strawberry tissue was homogenized with 5 mL of distilled water and 5 mL of saturated NaCl. The homogenate was transferred to two 20 mL

vials and capped. Samples were heated at 40 ^{o}C for 20 min. After incubation, a 1 mL headspace gas sample was taken and injected to a gas chromatograph model 5890 (Hewlett-Packard, Wilmington, DEL) equipped with a 15 m x 0.53 mm Stabilwax (Resteck, Bellefonte, PA) wide bore capillary column (1.0 μm coating thickness), and attached to a Hewlett-Packard 3396A integrator as described by Fernandez-Trujillo et al. (1999).

5.2.6 Statistical analysis

All data were subjected to the General Linear Model (GLM) analysis of variance using the MINITAB software release 15 (Minitab Inc., State College, PA). All means are average of five replicates. Factors used for analysis were cultivar, storage time, and atmosphere storage.

5.3 RESULTS

5.3.1 Fruit color

During fruit ripening, the overall brightness (L*) and Hue angle (H^{o}) decreased, but the decline was slower in CO_2 treated than air treated fruit (Table 5.1). Chroma value (C*) increased during storage at 2^{o}C, but in 'Allstar' and 'Northeaster', the increase was slightly greater in CO_2 treated fruit than air treated fruit.

5.3.2 Fermentation product

Acetaldehyde concentrations in the cultivar varied over time with no consistent patterns between air and CO_2 treatments or distinct differences among cultivars (Figure 5.1). In contrast, ethanol and ethyl acetate concentrations remained low during storage in both air and CO_2 storage of all cultivars except 'Jewel'.

5.3.3 GABA metabolism

‘Allstar’ and ‘Earliglow’ had the lowest GABA concentrations at harvest, being 0.15 and 0.15 mmol kg^{-1}, respectively (Figure 5.2). The highest GABA levels were detected in ‘Jewel’ with an average of 0.35 mmol kg^{-1}, while that of

Table 5.1 Brightness (L*), chroma (C*) and hue angle (h^o) of 'Allstar', 'Earliglow', 'Jewel', and 'Northeaster' strawberry fruit stored in air or 20% CO_2 at 2 ^oC for 12 days. Each mean is the average of 5 replicates of 5 fruit.

Days	Allstar		Earliglow		Jewel		Northeaster	
	Air	20% CO_2	Air	20% CO_2	Air	20% CO_2	Air	20% CO_2
Brightness (L*)								
0	46.9	46.9	46.7	46.7	44.7	44.7	45.5	45.5
3	43.0	44.1	44.2	43.2	39.4	40.3	38.7	42.3
6	41.4	43.7	44.5	43.6	38.9	40.5	38.3	42.2
9	43.3	43.6	44.1	43.5	39.3	40.7	38.7	42.6
12	41.5	43.9	42.3	44.0	38.2	40.9	38.2	42.6
LSD (P = 0.05)		8.56		1.55		1.35		1.36
Chroma (C*)								
0	46.9	46.9	45.6	45.6	45.8	45.8	43.9	43.9
3	48.9	47.6	48.9	49.4	43.5	44.1	46.0	47.2
6	49.7	49.3	47.0	48.4	44.8	46.6	46.5	48.7
9	47.5	48.6	48.9	49.0	47.6	47.6	45.4	46.4
12	49.1	50.1	51.5	51.1	48.7	48.9	46.1	48.8
LSD (P = 0.05)		1.16		1.0		1.51		1.55
Hue angle (h^o)								
0	45.1	45.1	45.7	45.7	47.8	47.8	45.1	45.1
3	43.5	45.0	45.4	44.7	44.0	44.7	40.8	43.9
6	42.1	44.4	45.3	44.3	44.0	45.6	40.3	43.3
9	41.5	41.5	44.6	45.4	41.8	44.4	39.0	41.5
12	41.1	42.9	42.1	43.8	40.7	44.5	39.4	42.7
LSD (P = 0.05)		2.14		1.95		1.58		1.53

Figure 5.1 Acetaldehyde, ethanol and ethyl acetate concentrations in 'Allstar', 'Earliglow', 'Jewel', and 'Northeaster' strawberry fruit stored in air or 20% CO_2 at 2 ^{o}C. Standard errors are shown by vertical bars when they exceed the size of the symbol, while the LSD bar (P=0.05) is shown in the upper left region of each figure. For acetaldehyde, cultivar (c), day (d), c x d, d x treatment (t), c x t, and c x d x t were significant at P <0.001, while t was significant at P = 0.012. For ethanol and ethyl acetate, all factors and interactions were significant at P <0.001.

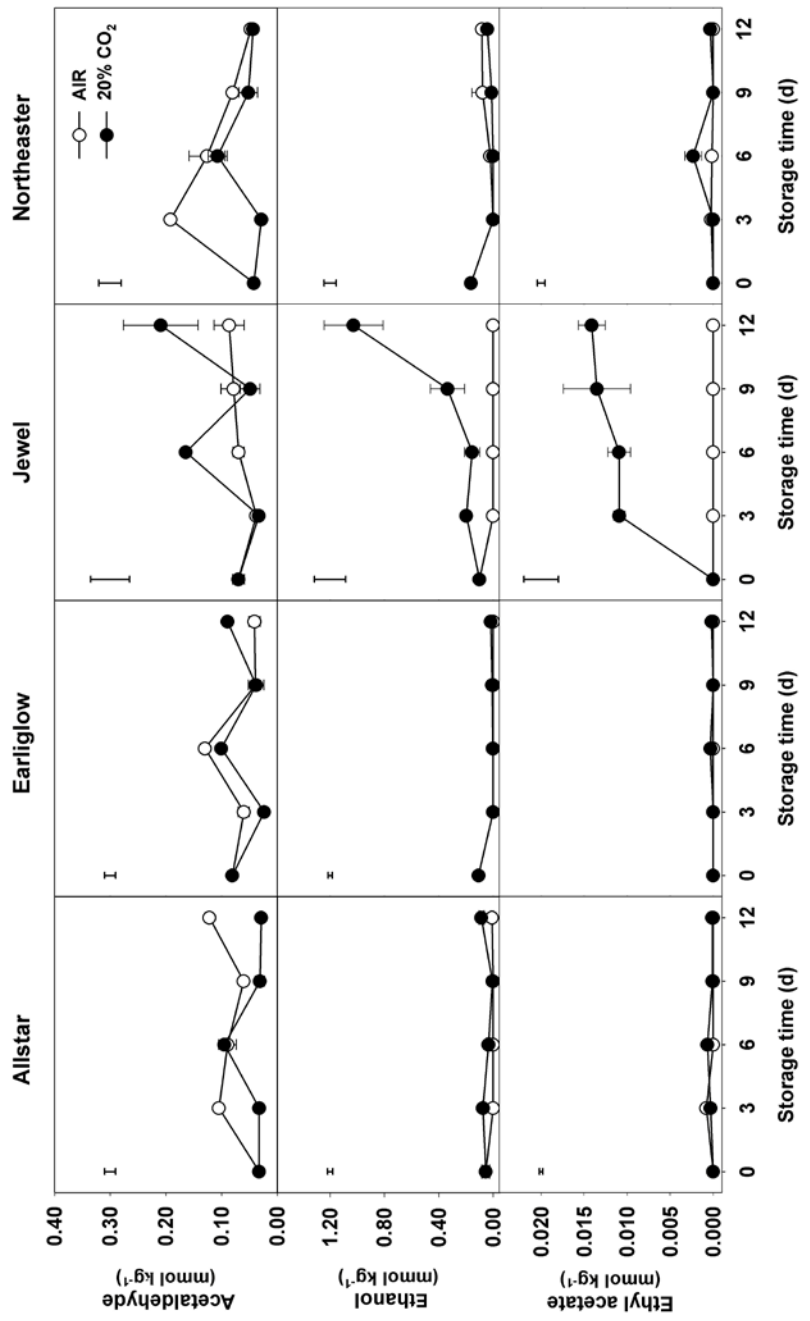
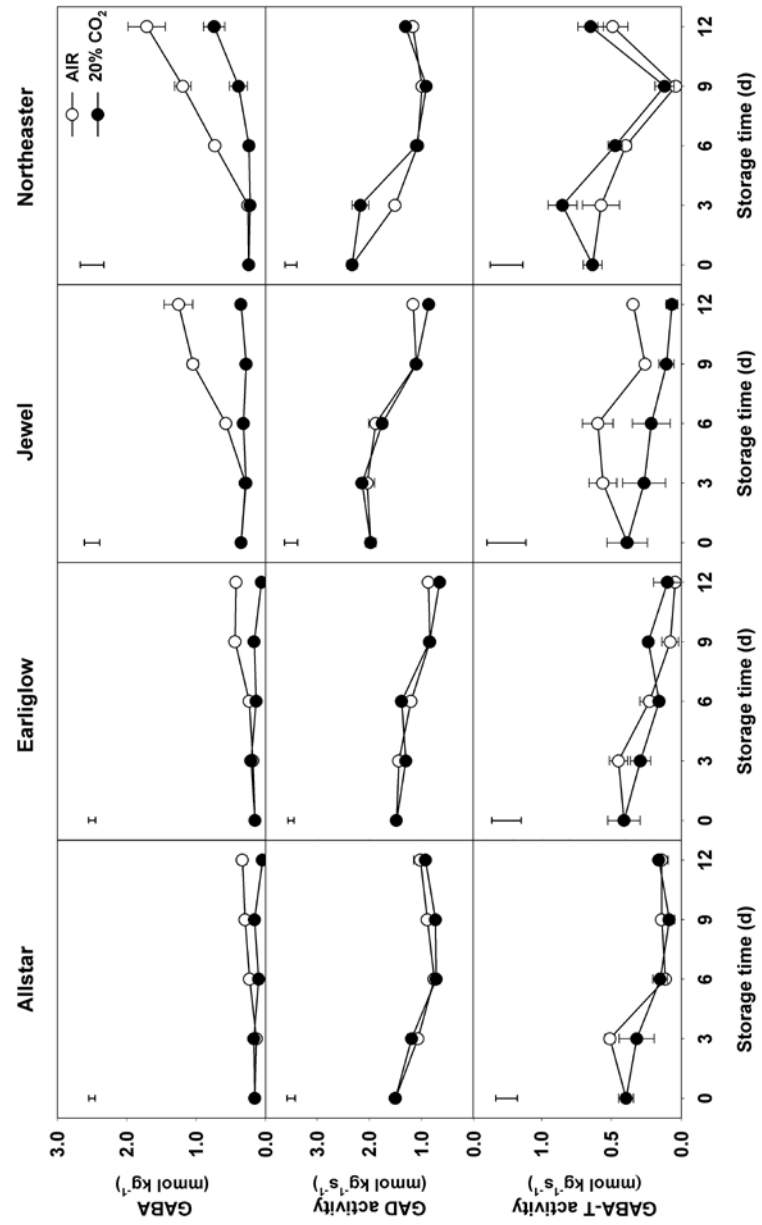

Allstar
Earliglow
Jewel
Northeaster
AIR
20% CO2
Acetaldehyde (mmol kg-1)
Ethanol (mmol kg-1)
Ethyl acetate (mmol kg-1)
Storage time (d)
0.40
0.30
0.20
0.10
0.00
1.20
0.80
0.40
0.00
0.020
0.015
0.010
0.005
0.000
0
3
6
9
12

Figure 5.2 GABA concentration, and specific activity of GAD (based on GABA production per protein mass) and GABA-T (based on alanine production per preotein mass) in 'Allstar', 'Earliglow', 'Jewel', and 'Northeaster' strawberry fruit stored in air or 20% CO_2 at 2 ^{o}C. Standard errors are shown by vertical bars when they exceed the size of the symbol, while the LSD bar (P = 0.05) is shown in the upper left region of each figure. For GABA, cultivar (c), day (d), treatment (t), c x d, d x t, and c x t were significant at $P < 0.001$, while c x d x t was significant at $P = 0.004$. For GAD activity, c, d, c x d, d x t, and c x d x t were significant at $P < 0.001$, while c x t was significant at $P = 0.003$. For GABA-T activity, c, d, c x d, and c x t were significant at $P < 0.001$. No other differences were significant.


Allstar
Earliglow
Jewel
Northeaster
AIR
20% CO2
GABA (mmol kg-1)
GAD activity (mmol kg-1s-1)
GABA-T activity (mmol kg-1s-1)
Storage time (d)
0
3
6
9
12
0.0
1.0
2.0
3.0
0.5

'Northeaster' was 0.24 mmol kg^{-1}. GABA concentrations in 'Allstar' and 'Earliglow' decreased during storage in air, but they remained unchanged in 'Jewel' and increased in 'Northeaster'. GABA concentrations in CO_2 treated fruit increased by 2.2-fold in 'Allstar', 2.8-fold in' Earliglow', 3.6-fold in 'Jewel', and 7.1-fold in 'Northeaster'. At the end of storage, GABA concentrations in 'Jewel' and 'Northeaster' were 3.5-fold and 2.3-fold higher in CO_2 treated fruit, respectively, than in air treated fruit.

At harvest, GAD activity was lower in 'Allstar' and 'Earliglow' than in 'Jewel' and 'Northeaster' (Figure 5.2). Enzyme activity decreased during storage and there were no significant differences between the activity of air treated and CO_2 treated fruit of any cultivar.

'Northeaster' had the highest GABA-T activity among the four cultivars at harvest (Figure 5.2). Enzyme activity in 'Allstar' and 'Earliglow' decreased by the end of storage and CO_2 treatment had no effect on the activity. In 'Jewel' fruit, the enzyme activity increased in air treated fruit during the first 6 d of storage and then declined, while CO_2 treatment decreased the enzyme activity by 5.8-fold. In 'Northeaster', the activity increased initially in CO_2 treated fruit but then activity decreased in fruit of both treatments until day 9 before then increasing. However, differences in enzyme activity between the two treatments were not significant.

5.4 DISCUSSION

Although fruit color changed to a small extent over the 12 d storage period, fruit slowly became darker and redder, as indicated by lower values of brightness and hue angle. These changes were slowed by CO_2 treatment, which is well known to inhibit strawberry ripening (Holcroft and Kader, 1999; Harker et al., 2000; Almenar et al., 2006).

Many volatile compounds have been identified in strawberries that contribute to strawberry flavor, and volatile profiles are different among cultivars (Yamashita et al., 1976; Zabetakis and Holden, 1997; Hakala et al., 2002). Acetaldehyde, ethanol, and ethyl acetate are among major volatile compounds that are normally found in ripe strawberries. However, over production of these fermentative compounds may cause off-flavor in the fruit and strawberry fruit vary in responses to elevated CO2 concentrations during storage (Fernandez-Trujillo et al., 1999; Watkins et al., 1999; Ponce-Valadez and Watkins, 2008). 'Jewel' was among the sensitive cultivars that accumulated ethanol and acetaldehyde during treatment with 20% CO_2 while others such as 'Earliglow' did not (Watkins et al., 1999). In the present study, we found that 'Jewel' accumulated ethanol and ethyl acetate during 20% CO_2 storage, while changes in other cultivars were generally small. The results indicate that the fermentative metabolism is different in each of the cultivars.

GABA accumulated in several fruit types in response to high CO_2 or low O_2 and high CO_2 conditions (Merodio et al., 1998; Makino et al., 2008; Pedreschi et al., 2009). In strawberry fruit, GABA accumulation occurred in CO_2 treated fruit of all cultivars, but to a much greater extent in 'Jewel' and 'Northeaster' than in 'Allstar' and 'Earliglow'. However, differences in GABA accumulation between 'Jewel' and 'Northeaster' were not evident. A lack of correlation between GABA and fermentation product accumulation in 'Northeaster' does not support a hypothesis that GABA is associated with high CO_2 stress in strawberry fruit. The time taken for GABA accumulation to occur in response to stresses varies from seconds to days depending on stress and plant system (Kinnersley and Turano, 2000). It is notable that effects of CO_2 on GABA in strawberry fruit were not evident until greater than 3 d of exposure, suggesting that a delayed response to stress in this system. Similar results were found in tomatoes in which significant higher GABA concentrations were detected 4 d after

modified atmosphere storages (Makino et al., 2008). However, changes could be occurring within individual tissues or components that are not easily detected by whole tissue assays.

CO_2 treatment did not affect GAD and GABA-T activities except for a strong reduction in GABA-T activity in high CO_2 treated fruit of 'Jewel'. Although CO_2 did not affect the activity of GAD enzyme, a decline in the rate of GABA degradation as indicated by GABA-T activity could contribute to the net increase in GABA. However, GABA accumulation in 'Northeaster' is not associated with effects on GABA-T activity. Inhibition of some enzymes in the TCA cycle under high CO_2 stress causes accumulation of succinate (Frenkel and Patterson, 1973; Knee, 1973; Shipway and Bramlage, 1973) which in turn blocks GABA-T. GAD and GABA-T are the key enzymes in the regulation of GABA levels (Bouche and Fromm, 2004), but GABA accumulation can possibly be regulated at other levels including transcriptional/translational and post-translational levels. In addition, availability of glutamate may be another factor affecting GABA production under high CO_2 treatments; Ponce-Valadez et al.(2008).found that a gene encoding glutamate dehydrogenase was suppressed under elevated CO_2 conditions in a sensitive strawberry cultivar but not in a tolerant cultivar. The results of the present study show that GABA accumulation is induced by CO_2 treatment of strawberry fruit, but no strong association between GABA accumulation and tolerance to high CO_2 based on accumulation of fermentation products was revealed.

REFERENCES

Almenar, E., Hernandez-Munoz, P., Lagaron, J.M., Catala, R., Gavara, R., 2006. Controlled atmosphere storage of wild strawberry fruit (*Fragaria vesca* L.). J. Agric. Food Chem. 54, 86-91.

Ansari, M.I., Lee, R.H., Chen, S.C.G., 2005. A novel senescence-associated gene encoding γ-aminobutyric acid (GABA):pyruvate transaminase is upregulated during rice leaf senescence. Physiol. Plant. 123, 1-8.

Aurisano, N., Bertani, A., Reggiani, R., 1995. Involvement of calcium and calmodulin in protein and amino-acid metabolism in rice roots under anoxia. Plant Cell Physiol. 36, 1525-1529.

Balazs, R., Machiyama, Y., Hammond, B.J., Julian, T., Richter, D., 1970. The operation of the γ-aminobutyrate bypath of the tricarboxylic acid cycle in the brain tissue *in vitro*. Biochem. J. 116, 445-467.

Bartyzel, I., Pelczar, K., Paszkowski, A., 2003. Functioning of the γ-aminobutyrate pathway in wheat seedlings affected by osmotic stress. Biol. Plant. 47, 221-225.

Bouche, N., Lacombe, B., Fromm, H., 2003. GABA signaling: a conserved and ubiquitous mechanism. Trends Cell Biol. 13, 607-610.

Bouche, N., Fromm, H., 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9, 110-115.

Bown, A.W., Shelp, B.J., 1989. The metabolism and physiological roles of 4-aminobutyric acid. Biochem. J. 8, 21-25.

Breitkreuza, K.E., Shelp, B.J., Fischer, W.N., Schwacke, R., Rentsch, D., 1999. Identification and characterization of GABA, proline and quaternary

ammonium compound transporters from *Arabidopsis thaliana*. FEBS-Letters 450, 280-284.

Fernandez-Trujillo, J.P., Nock, J.F., Watkins, C.B., 1999. Fermentative metabolism and organic acid concentrations in fruit of selected strawberry cultivars with different tolerances to carbon dioxide. J. Am. Soc. Hort. Sci. 124 (6), 696-701.

Fernandez-Trujillo, J.P., Nock, J.F., Watkins, C.B., 2007. Antioxidant enzyme activities in strawberry fruit exposed to high carbon dioxide atmospheres during cold storage. Food Chem. 104, 1425-1429.

Franck, C., Lammertyn, J., Tri Ho, Q., Verboven, P., Verlinden, B., Nicolaï, B.M., 2007. Browning disorders in pear fruit. Postharvest Biol. Technol. 43, 1-13.

Frenkel, C., Patterson, M.E., 1973. Effect of carbon dioxide on activity of succinic dehydrogenase in 'Bartlett' pears during cold storage. HortScience 8, 395-396.

Hakala, M.A., Lapvetelainen, A.T., Kallio, H.P., 2002. Volatile compounds of selected strawberry varieties analyzed by purge-and trap headspace GC-MS. J. Agric. Food Chem. 50, 1133-1142.

Harker, F.R., Elgar, H.J., Watkins, C.B., Jackson, P.J., Hallett, I.C., 2000. Physical and mechanical changes in strawberry fruit after high carbon dioxide treatments. Postharvest Biol. Technol. 19, 139-146.

Holcroft, D.M., Kader, A.A., 1999. Controlled atmosphere-induced changes in pH and organic acid metabolism may affect color of stored strawberry fruit. Postharvest Biol. Technol. 17, 19-32.

Kinnersley, A.M., Turano, F.J., 2000. Gamma aminobutyric acid (GABA) and plant responses to stress. Crit. Rev. Plant Sci. 19, 479-509.

Knee, M., 1973. Effects of controlled atmosphere storgae on respiratory metabolism of apple fruit tissue. J. Sci. Food Agric. 24, 1289-1298.

Little, A.C., 1975. Off on a tangent. J. Food Sci. 40, 410-411.

MacGregor, K.B., Shelp, B.J., Peiris, S., Bown, A.W., 2003. Overexpression of glutamic acid decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. J. Chem. Ecol. 29, 2177-2182.

Makino, Y., Soga, N., Oshita, S., Kawagoe, Y., Tanaka, A., 2008. Stimulation of γ-aminobutyric acid production in vine-ripe tomato (*Lycopersicon esculentum* Mill.) fruits under modified atmospheres. J. Agri. Food Chem. 56, 7189-7193.

Mayer, R.R., Cherry, J.L., Rhodes, D., 1990. Effects of heat shock on amino acid metabolism of cowpea cells. Phytochemistry 94, 796-810.

McLean, M.D., Yevtushenko, D.P., Deschene, A., Van Cauwenberghe, O.R., Makhmoudova, A., Potter, J.W., Bown, A.W., Shelp, B.J., 2003. Overexpression of glutamic acid decarboxylase in transgenic tobacco plants confers resistance to the northern root-knot nematode. Mol. Breed. 11, 277-285.

Merodio, C., Munoz, M.T., Cura, B.D., Buitrago, D., Escribano, M.I., 1998. Effect of high CO_2 level on the titres of γ-aminobutyric acid, total polyamines and some pathogenesis-related proteins in cherimoya fruit stored at low temperature. J. Exp. Bot. 49, 1339-1347.

Mitcham, E.J., 2004. Strawberry. In: Gross, K.C., Wang, C.Y., Saltveit, M.E. (Eds.), The commercial storage of fruits, vegetables, and florist and nursery crops. U.S. Dept. Agric., Agric. Handbook 66, ://www.ba.ars.usda.gov/hb66/index.html.

Pedreschi, R., Franck, C., Lammerteyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123-130.

Ponce-Valadez, M., Fellman, S.M., Giovannoni, J., Gan, S., Watkins, C.B., 2008. Differential fruit gene expression in two strawberry cultivars in response to

elevated CO_2 during storage revealed by a heterologous fruit microarray approach. Postharvest Biol. Technol. 51, 131-140.

Ponce-Valadez, M., Watkins, C.B., 2008. Fermentation and malate metabolism in response to elevated CO_2 concentrations in two strawberry cultivars. Physiol. Plantarum 134, 121-133.

Saito, T., Matsukura, C., Sugiyama, M., Watahiki, A., Ohshima, I., Iijima, Y., Konishi, C., Fujii, T., Inai, S., Fukuda, N., Nishimura, S., Ezura, H., 2008. Screening for γ-aminobutyric acid (GABA)-rich tomato varieties. J. Japan. Soc. Hort. Sci. 77, 242-250.

Shelp, B.J., Bown, A.W., McLean, M.D., 1999. Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446-452.

Shipway, M.R., Bramlage, W.J., 1973. Effects of carbon dioxide on activity of apple mitochondria. Plant Physiol. 51, 1095–1098.

Streeter, J.G., Thompson, J.F., 1972. *In vivo* and *in vitro* studies on γ-aminobutyric acid metabolism with the radish plant (*Raphanus sativus* L.). Plant Physiol 49, 572-584.

Tsushida, T., Murai, T., 1987. Conservation of glutamic acid to γ-aminobutyric acid in tea leaves under anaerobic conditions. Agric. Biol. Chem. 51, 2865-2871.

Wallace, W., Secor, J., Schrader, L.E., 1984. Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol 75, 170-175.

Watkins, C.B., Manzano-Mendez, J.E., Nock, J.F., Zhang, J., Maloney, K.E., 1999. Cultivar variation in response of strawberry fruit to high carbon dioxide treatments. J. Sci. Food Agric. 79, 886-890.

Yamashita, I., Nemoto, Y., Yoshikawa, S., 1976. Formation of volatile alcohols and esters from aldehydes in strawberries. Phytochemistry 15, 1633-1637.

Zabetakis, I., Holden, M., 1997. Strawberry flavor: analysis and biosynthesis. J. Sci. Food Agric. 74, 421-434.

Zhang, G., Bown, A.W., 1997. The rapid determination of γ-aminobutyric acid. Phytochemistry 44, 1007-1009.

Zushi, K., Matsuzoe, N., 2007. Salt stress-enhanced γ-aminobutyric acid (GABA) in tomato fruit. Acta Hort 761, 431-435.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

γ-Aminobutyric acid (GABA) metabolism in tomatoes exposed to chilling temperarures or elevated CO_2, and strawberries exposed to elevated CO_2, was investigated to reveal effects of postharvest stresses. To investigate metabolism, GABA production, the activities of GABA shunt enzymes, and the expression of genes encoding enzymes in GABA production and degradation, was studied. In addition, the work with cold stored tomatoes included antioxidant metabolism in the fruit.

As shown by results from tomato experiments (CHAPTER 3 and 4), more tolerant fruit to chilling and high CO_2 stresses do not accumulate GABA in response to these stresses. Instead, GABA may be recycled through the GABA shunt, suggesting that its role in response to stress, if any, may involve the maintenance of the carbon flux and redox balance. In contrast to tolerant lines, GABA degradation was altered in fruit with more sensitivity to stresses as indicated by suppression of GABA-T activity and expression of some genes encoding GABA degradation enzymes. As described in the CHAPTER 3, the ability of tissues to scavenge reactive oxygen species was higher in tolerant than intolerant lines as indicated by higher ascorbate peroxidase (APX) activity during cold storage and ripening, higher peroxidase (POX) activity during ripening, and higher gene expression of a gene encoding superoxide dismutase (SOD) during cold storage.

The study in strawberry (CHAPTER 5) indicated that elevated CO_2 induced GABA production in strawberry fruit, but lack of correlation between GABA accumulation and sensitivity to stress suggests an uncertain role of GABA in response to high CO_2 in strawberry fruit.

In summary, this study has found that GABA accumulation is not an immediate response to postharvest stresses, and it is still uncertain whether or not these responses are associated with the sensitivity of the fruit to stresses. It is well known that glutamate is a major precursor for GABA production, but the pathway using putrescine as a substrate also occurs in plants. Putrescine is another compound that also accumulates under stress conditions and is regulated by cytosolic pH (Young and Galston, 1983; Hiatt and Malmberg, 1988; Ye et al., 1997). Also, GABA production is limited by the amount of the precursor available. Thus, glutamate and putrescine concentrations should be analyzed to specify substrate for GABA production under stresses. Other related amino acids and intermediates such as alanine, succinate, succinic semialdehyde (SSA), γ-hydroxybutyrate (GHB) should also be determined in order to see a broarder effect of the stresses.

Three GABA-T isozymes have been identified; puruvate-dependent, α-ketoglutarate-dependent, and glyoxylate-dependent GABA-Ts (Clark et al., 2009). There is still some controversy whether α-ketoglutarate-dependent GABA-T exists in tomato fruit (Akihiro et al., 2008; Clark et al., 2009). Determination of enzyme activity of all isozymes as well as isolation and characterization of genes encoding GABA-Ts in tomato fruit should be conducted to see how the degradation steps are regulated. Also, the results from the CHAPTERS 3 and 4 showed induction of *SSR* genes by stresses, suggesting a possible involvement of this enzyme in controlling GABA concentrations. Enzyme activity assays of SSADH and SSR should also be investigated. As shown in CHAPTERS 3 and 4, the net GABA levels seem to be regulated by degradation of GABA; knockout mutants of genes encoding fruit specific GABA-Ts, SSADH(s), and SSR(s) in tomatoes may be useful to study their function under postharvest stresses.

In the strawberry, an inconsistent relationship between GABA accumulation under CO_2 treatment and sensitivity of the fruit to stress was found. To clarify the detailed mechanisms of GABA metabolism, gene expression of the GABA shunt enzymes and metabolic study to identify all intermediates are needed to better understand how GABA metabolism is related to CO_2-induced stress.

GABA metabolism under stress conditions seems to be complex and may involve other metabolic processes such as antioxidant defense systems, the glycolate pathway, nitrogen metabolism and aldehyde detoxification. To further investigate the detailed transcriptional regulation of tolerant and sensitive fruit under postharvest stresses, a microarray based method is suggested to deeper understand global change in plant metabolism. Integration of multiple microarray data produced from fruit with different stress sentivity to reveal changes of non-stress regulated and stress induced genes. Additionally, a time-course microarray may be useful to identify early and late responses of genes under stress conditions. To confirm microarray results, quantitative RT-PCR would be used to confirm microarray results of key relationships.

REFERENCES

Akihiro, T., Koike, S., Tani, R., Tominaga, T., Watanabe, S., Iijima, Y., Aoki, K., Shibata, D., Ashihara, H., Matsukura, C., Akama, K., Fujimura, T., Ezura, H., 2008. Biochemical mechanism on GABA accumulation during fruit development in tomato. Plant Cell Physiol. 49, 1378-1389.

Clark, S.M., Leo, R.D., Van Cauwenberghe, O.R., Mullen, R.T., Shelp, B.J., 2009. Subcellular localization and expression of multiple tomato γ-aminobutyrate transaminases that utilize both pyruvate and glyoxylate. J. Exp. Bot. 60, 3255-3267.

Hiatt, A., Malmberg, R.L., 1988. Utilization of putrescine in tobacco cell lines resistant to inhibitors of polyamine synthesis. Plant Physiol. 86, 441-446.

Ye, B., Müller, H.H., Zhang, J., Gressel, J., 1997. Constitutively elevated levels of putrescine and putrescine-cenerating enzymes correlated with oxidant stress resistance in *Conyza bonariensis* and wheat. Plant Physiol. 115, 1443-1451.

Young, N.D., Galston, A.W., 1983. Putrescine and acid stress. Plant Physiol. 71, 761-771.

APPENDIX

Surface pitting and decay incidence in tomato introgression lines stored for 3 weeks at 3 ^{o}C. Severity of surface pitting was evaluated a day after removal to 20 ^{o}C, while decay incidence was examined a week after removal to 20 ^{o}C. Values were the means of samples from 6 replicate fruit per line.

Introgression line	Pitting	Decay
IL 1-1	1.17 fedc	1.50 ebdhagcf
IL 1-1-2	1.00 fed	1.17 ebdhgcf
IL 1-4	1.17 fedc	1.33 ebdhagcf
IL 1-4-18	0.83 fed	1.17 ebdhgcf
IL 2-1	1.33 fedc	1.40 ebdhagcf
IL 2-1-1	0.33 fed	1.00 edhgcf
IL 2-2	1.33 fedc	0.50 ehgf
IL 2-3	0.83 fed	1.00 edhgcf
IL 2-4	0.00 f	0.00 h
IL 2-6	0.00 f	0.33 hgf
IL 2-6-5	0.50 fed	0.33 hgf
IL 3-1	0.20 fe	1.00 edhgcf
IL 3-2	1.50 fbedc	0.67 edhgf
IL 3-3	0.67 fed	0.00 h
IL 3-4	3.33 a	2.17 bdac
IL 3-5	1.00 fed	1.50 ebdhagcf
IL 4-1	0.33 fed	0.50 ehgf
IL 4-1-1	1.50 fbedc	1.00 edhgcf
IL 4-2	1.33 fedc	2.00 ebdac

Introgression line	Pitting	Decay
IL 4-3	1.80 bdc	2.33 bac
IL 4-3-2	0.50 fed	0.83 edhgcf
IL 5-1	1.33 fedc	1.17 ebdhgcf
IL 5-2	0.83 fed	1.00 edhgcf
IL 5-3	1.33 fedc	2.00 ebdac
IL 5-4	0.83 fed	0.00 h
IL 5-5	0.67 fed	0.83 edhgcf
IL 6-1	0.17 fe	0.50 ehgf
IL 6-2	0.33 fed	0.50 ehgf
IL 6-3	0.83 fed	0.50 ehgf
IL 6-4	0.83 fed	0.83 edhgcf
IL 7-1	0.83 fed	1.50 ebdhagcf
IL 7-2	1.17 fedc	1.33 ebdhagcf
IL 7-3	0.00 f	0.17 hg
IL 7-4	1.67 bedc	1.50 ebdhagcf
IL 7-4-1	2.83 ab	2.80 a
IL 7-5	1.33 fedc	1.50 ebdhagcf
IL 7-5-5	1.67 bedc	1.50 ebdhagcf
IL 8-1	1.00 fed	2.67 ab
IL 8-1-1	0.50 fed	1.83 ebdacf
IL 8-1-2	0.00 f	0.67 edhgf
IL 8-2	1.00 fed	1.60 edhgcf
IL 8-2-1	2.50 abc	2.00 ebdac
IL 8-3	0.67 fed	1.50 ebdhagcf

Introgression line	Pitting	Decay
IL 8-3-1	1.17 fedc	1.17 ebdhgcf
IL 9-1	0.50 fed	0.50 ehgf
IL 10-1-1	0.33 fed	1.00 edhgcf
IL 10-2	1.20 fedc	1.00 edhgcf
IL 10-2-2	1.17 fedc	1.00 edhgcf
IL 10-3	0.67 fed	0.17 hg
IL 11-1	0.33 fed	0.17 hg
IL 11-2	0.00 f	0.50 ehgf
IL 11-3	0.67 fed	0.67 edhgf
IL 11-4	1.17 fedc	1.83 ebdacf
IL 11-4-1	1.83 bdc	0.67 edhgf
IL 12-1	1.00 fed	1.00 edhgcf
IL 12-1-1	0.83 fed	0.33 hgf
IL 12-2	1.00 fed	1.00 edhgcf
IL 12-3	1.33 fedc	1.80 ebdacf
IL 12-3-1	0.50 fed	0.83 edhgcf
IL 12-4	1.60 bedc	1.80 ebdacf
IL 12-4-1	1.33 fedc	0.67 edhgf

Means followed by the same letter within columns are not significantly different from each other according to the Duncan's multiple range tests at the 5% level.